NITROGENOUS HETEROCYCLES: MODULATION OF CHEMICAL PROPERTIES, AND APPLICATIONS TO SYNTHETIC AND MEDICINAL CHEMISTRY

A thesis
submitted in partial fulfillment
of the requirements for the degree
of
Doctor of Philosophy in Chemistry
at the
University of Canterbury
by
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University of Canterbury
2003

WORK IN THIS THESIS HAS APPEARED IN THE FOLLOWING PUBLICATIONS

Martyn, D. C.; Vernall, A. J.; Clark, B. M.; Abell, A. D.
J. Chem. Soc., Org. Biomol. Chem. **2003**, *1*, 2103.

Abell, A. D.; Martyn, D. C.; May, B. C. H.; Nabbs, B. K.
Tetrahedron Lett. **2002**, *43*, 3673.

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ABSTRACT

This thesis examines ways in which the chemical reactivity of benzimidazole-, pyrrole- or pyrrolizinone-based heterocycles can be modulated. The latent reactivity of these systems has been used to develop enzyme inhibitors, antitumour compounds and a novel amino acid derivatisation technique.

Chapter One introduces the concept of modulated chemical reactivity, with illustrative examples from biochemistry, synthesis and medicinal chemistry. The application of latent reactivity towards the inhibition of serine proteases, a class of proteolytic enzymes, is then reviewed. Two inhibitor classes are discussed, the first of which require an inorganic cofactor, *e.g.* a metal ion, for enhanced activity. Secondly, mechanism-based inhibitors, which rely on activation by the protease to covalently derivatise the enzyme, are discussed. The application of latent reactivity to effect DNA alkylation, and hence inhibit tumour cell growth, is also reviewed. This section places particular emphasis on alkylators that are attached to an intercalator to enhance their affinity for DNA.

Chapter Two describes the inhibition of α-chymotrypsin, a representative serine protease, by compounds that were proposed to exhibit enhanced activity upon the coordination of Zn^{2+}. The attempted synthesis of the putative inhibitors **2.1a-b** was hindered by the instability of the diamine precursors **2.2a-b**. The model compound **2.1c** was prepared, however this compound was prone to deuteration and oxidation at its methylene bridge. Benzimidazoles **2.14a-c** were subsequently prepared, in addition to the dihydrochloride salt of **2.14a**. These compounds were moderate to weak inhibitors of α-chymotrypsin, and no Zn^{2+}-mediated enhancement of inhibition was observed. Benzimidazole **2.14b** was shown to coordinate $Zn(OAc)_2$ by ^1H NMR spectroscopy, which illustrated that the lack of Zn^{2+}-enhanced inhibition of α-chymotrypsin by compounds of type **2.14** did not result from an inability to complex metal ions.

Chapter Three describes the inhibition of α-chymotrypsin by a new series of C2-acyl-C5-(hydroxyalkyl)pyrroles. These compounds are postulated to be mechanism-based inhibitors. The pyrrole-based peptidomimetics **3.13a** and **3.14a** were prepared and assayed against α-chymotrypsin. Minimal difference in activity was observed between these compounds, despite the bulky hydrophobic moiety at C5 of **3.14a**, which was proposed to bind more tightly to the S_1 subsite than the 5-(hydroxymethyl) group of **3.13a**. Mass spectrometry of **3.13a** incubated with α-chymotrypsin suggested that inhibition was non-covalent in nature. Modifications at the C2 position of **3.13a** led to the preparation of **3.13b**, **3.24** and **3.25**, of which the latter was the optimal inhibitor, and an IC_{50} value of 108 μM was determined for this compound. The attempted solid phase synthesis of **3.13a** was ineffectual due to the instability of the 5-(hydroxymethyl)pyrrole moiety to TFA. Solid phase synthesis of a 5-formylpyrrole precursor to **3.13a** gave the desired product **3.36** as an inseparable mixture with pyrrole acid **3.16b**. Solution phase synthesis of the 5-(hydroxymethyl)pyrrole **3.35**, which possessed a carboxylic acid moiety, afforded an unstable compound.

Chapter Four details a novel derivatisation method for amino acids, whereby the amine is first "capped" by pyrrole acid **3.16b**. Subsequent reaction with hydrocinnamoyl chloride releases the latent spectrophotometric properties of the pyrrole-amino acid adduct. A previous study afforded pyrrolizinones **4.11a-c**, and this study expanded the range of pyrrolizinones available for analysis to include **4.11d-i** and **4.17a-b**. Initial UV-vis spectroscopic analysis revealed that methanol degraded pyrrolizinone **4.11e**. UV-vis spectroscopy of **4.11a-i/4.17a-b** in acetonitrile revealed that the variable R^1 and R^2 groups did not affect the λ_{max} and ε values in a systematic manner. Analysis of the LRMS fragmentation patterns for **4.11a-i/4.17a-b**, and their pyrrole precursors **3.18a-i/3.29a-b**, revealed that a product ion was generated whose mass was dependent on the R^1 group. The abundance of the diagnostic product ion was dependent on the nature of R^1 and R^2. Fragmentation was increased by the technique of electron impact, compared to electrospray ionisation.

Chapter Five describes the application of 5-(hydroxymethyl)pyrroles towards the development of DNA alkylators with latent reactivity. Attachment of an intercalator to the pyrrole moiety via an alkyl linker was proposed to increase cytotoxicity. Model studies afforded **5.21** (naphthyl-based) and **5.31** (quinolyl-based), however the synthetic methodology was not amenable to compounds that incorporated anthryl or acridine moieties, *i.e.* "true intercalators". Compounds **5.21** and **5.31** displayed moderate activity against the P388 cancer cell line. The 5-(hydroxymethyl)pyrrole substructure analogue (**5.41**) elicited no activity in the P388 assay. Compound **5.31** did not react with aniline, which suggested that compounds of this type would not effect DNA alkylation *in vivo*. A DNA binding assay revealed that **5.31** had a low affinity for the genetic material, which rationalised its moderate P388 activity. Compounds **5.21** and **5.31** displayed minimal antiviral and antimicrobial activity.

ABBREVIATIONS

λ_{max}	absorbance maximum
ε	extinction coefficient
AB_q	AB quartet (in NMR)
BABIM	bis(5-amidino-1*H*-benzimidazolyl)methane
BOP	benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexa-fluorophosphate
BOP-Cl	bis(2-oxo-3-oxazolidinyl)phosphinic chloride
CIGAR	constant time inverse-detected gradient accordian rescaled long-range HMBC (in NMR)
COSY	correlation spectroscopy
d	doublet (in NMR)
Da	Dalton
DIBALH	diisobutylaluminium hydride
DIEA	*N*,*N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribosenucleic acid
dt	double of triplets (in NMR)
EDCI	1-[3-(dimethylamino)propyl]-3-carbodiimide hydrochloride
EDTA	ethylenediamine tetraacetate
EI	electron impact ionisation (in mass spectrometry)
ESI	electrospray ionisation (in mass spectrometry)
EWG	electron-withdrawing group
FTIR	Fourier transform infrared
h	hour(s)
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond correlation (in NMR)

HOBT	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HSQC	heteronuclear single quantum correlation (in NMR)
Hz	hertz (in NMR)
IC_{50}	inhibitor concentration that decreases enzyme activity by 50%
ID_{50}	test compound concentration that reduces growth by 50%
J	coupling constant (in NMR)
K_i	enzyme-inhibitor dissociation constant
LAH	lithium aluminium hydride
LCMS	liquid chromatography mass spectrometry
LRMS	low resolution mass spectrometry
m	multiplet (in NMR)
Micro.	microanalysis
min	minute(s)
mp	melting point
NMR	nuclear magnetic resonance
PPA	polyphosphoric acid
ppm	parts per million
q	quartet (in NMR)
RNA	ribosenucleic acid
r.t.	room temperature
s	singlet (in NMR)
s(br)	broad singlet (in NMR)
t	triplet (in NMR)
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
UV	ultraviolet
UV-vis	ultraviolet-visible

ACKNOWLEDGMENTS

First and foremost, I would like to thank Associate Professor Andrew Abell for his help and guidance over the last four years. Not only did his research projects interest and stimulate me, but his style of supervision has allowed me to grow as a researcher. The countless hours you have spent reading my thesis, reports and scholarship applications have been invaluable to me.

Thanks to all the academic and technical staff I have pestered over the last few years. In particular, Rewi Thompson and Professor John Blunt (NMR), Bruce Clark (mass spectrometry), Professor Ward Robinson and Dr Jan Wikaira (X-ray crystallography), and Gill Barnes (biological assays) have contributed to the material in this thesis.

To the students I met in this department, thanks for the laughs and good times, especially the netballers, footballers and the "beer o'clock" attendees – you have made sure that my time here has been enjoyable. The Abell Group has been great bunch of people to work with over the years, there are too many to thank, however I think it is time to go when I am being continually mocked about my age! Best of luck to all of you, it will be great to hear of your progress over the years. Florian, keep the girls in line. And the writing up process was made bearable due to the use of Sarah Lundy's discman.

To my "real world" friends, your banter and friendship has been fantastic, but now seeing I am "Dr Martyn" you will have to give me the respect I deserve.

I would like to thank my parents Kay and Alan for their support, and also to my extended family for their words of encouragement. Maybe when you get to read my thesis you will finally find out what I have been mucking around on for the last few years!

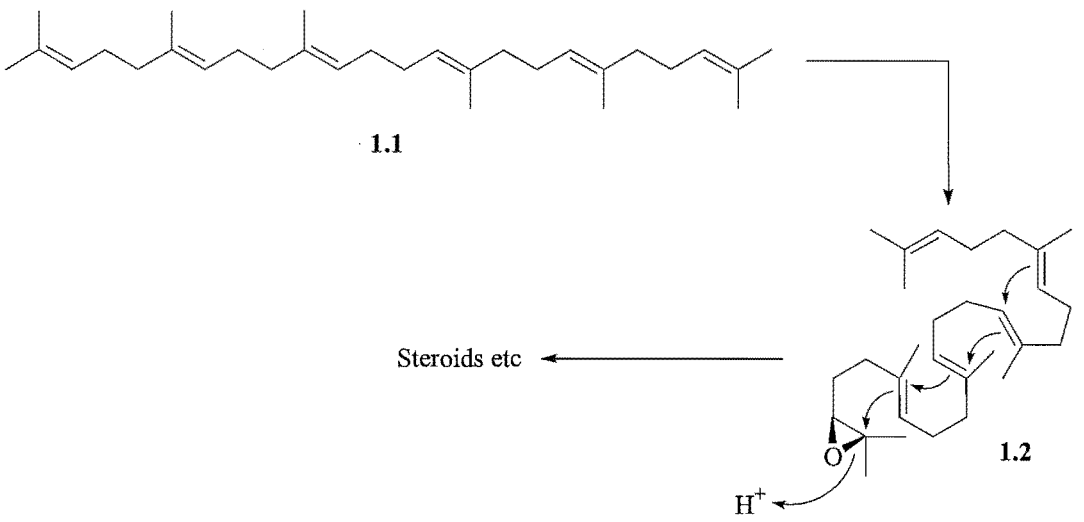
And finally, thanks to Malina for her love and support, especially during the thesis-writing process. And I really appreciate your efforts in proof-reading this epic!

CHAPTER ONE

INTRODUCTION

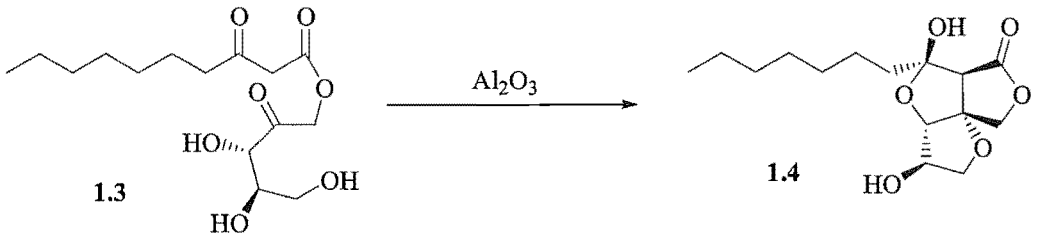
1.1 OVERVIEW

Selective and controlled manipulation of the chemical and physical properties possessed by a molecule is of extreme importance in many areas of chemistry and biology. Without the modulation of reactivity, a molecule that is too reactive may form undesired side products. Conversely, a chemically stable molecule may not undergo the desired transformation. Nature controls chemical reactivity to perform a wide array of essential reactions. An example is the formation of steroids from the acyclic precursor squalene (**1.1**, Scheme 1.1).[1] The reactivity inherent within the structure of **1.1** is released only upon the formation of squalene monoepoxide (**1.2**), after which cyclisation occurs.



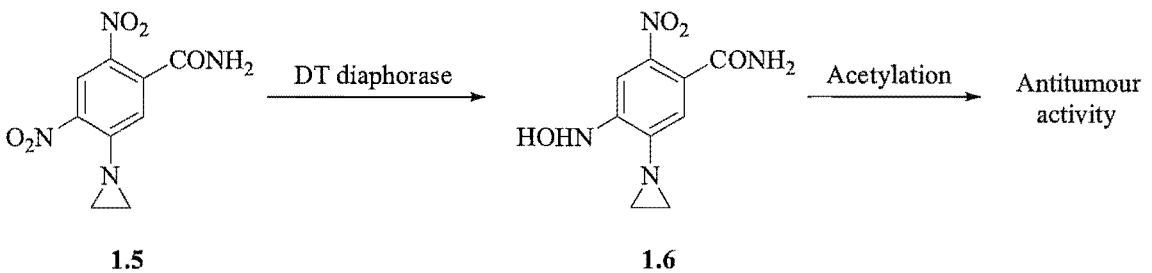
Scheme 1.1. Formation of steroids by the conversion of squalene (**1.1**) to squalene monoepoxide (**1.2**).

The concept of modulation of chemical reactivity is also evident in synthetic chemistry. The latent reactivity possessed by a synthetic intermediate often complements, or is an alternative to, the use of protecting groups to effect the desired timing and outcome of a reaction. One example is the exposure of **1.3** (Scheme 1.2), a D-xylulose-based intermediate, to basic alumina, which results in a triple cyclisation to give syringolide 2 (**1.4**) as a single stereoisomer.[2]



Scheme 1.2. Key cyclisation step in the synthesis of syringolide 2 (**1.4**).

The controlled release of chemical reactivity is also utilised by medicinal chemists. Highly specific interactions between a molecule and a particular biological target can be effected by unmasking the molecules latent reactivity. One example of such an activation process is illustrated in Scheme 1.3, whereby the benzamide prodrug **1.5** was selectively reduced at the 4-nitro position by DT diaphorase,[3] an enzyme expressed at high levels in the Walker 256 rat carcinoma, to the hydroxylamine **1.6**. Subsequent acetylation by thioesters such as acetyl-Coenzyme A further activates **1.6**, which allowed it to act as a cytotoxic agent towards cancerous cells.



Scheme 1.3. Activation of **1.5**, an antitumour prodrug.

An important application of latent reactivity within medicinal chemistry involves modulating the activity of enzymes as a result of specific interactions elicited by compounds that possess latent reactivity. An example of such a process developed in these laboratories is illustrated in Figure 1.1. Irradiating the *E* isomer of azobenzene **1.7** at approximately 350 nm gave (*Z*)-**1.7**, a two-fold more effective inhibitor of α-chymotrypsin.[4] The altered shape of the *Z* isomer resulted in tighter binding of **1.7** to the

enzyme active site, *i.e.* the azobenzene moiety is a photobiological switch that increases enzyme inhibition upon adopting the correct conformation.

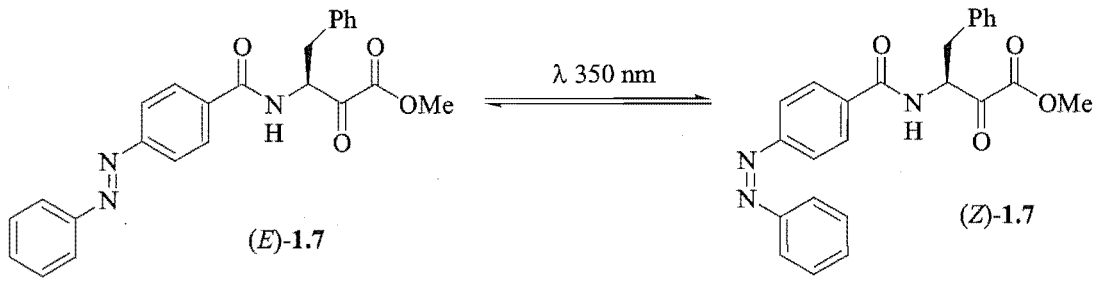
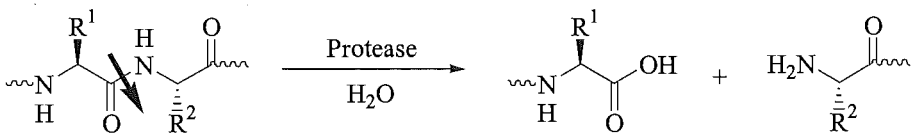


Figure 1.1. Photoisomerism of azobenzene **1.7**.

The remainder of this introduction is divided into two sections. The first discusses the inhibition of serine proteases, a subclass of proteolytic enzymes, by compounds whose activity is dependent on modulated chemical reactivity. The second section deals with compounds that act as anticancer agents by the alkylation of DNA only upon the formation of reactive intermediates. This section also addresses how the affinity of alkylators for the genetic material is increased by incorporation of an intercalating moiety.

1.2 INHIBITION OF SERINE PROTEASES BY COMPOUNDS WITH CHEMICALLY MODULATED ACTIVITY

Proteases are a class of enzyme that catalyse the hydrolysis of peptide bonds found in proteins and polypeptides (represented by **1.8** in Scheme 1.4) to give an *N*-terminal fragment (**1.9**) and a *C*-terminal fragment (**1.10**).[5]



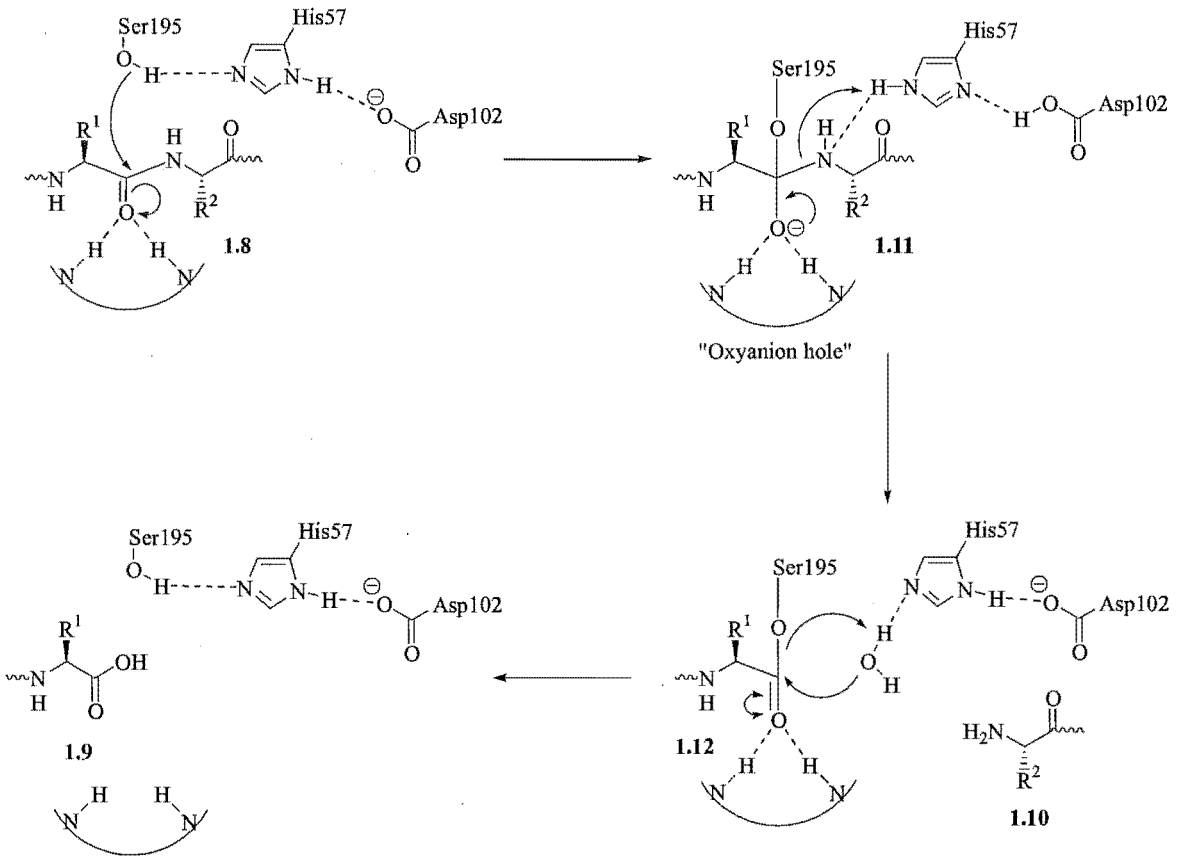
1.8 **1.9** **1.10**

Scheme 1.4. Hydrolysis of the peptide **1.8** by a protease gives fragments **1.9** and **1.10**. The point of cleavage is represented by a bold arrow.

There are four subclasses of proteases; serine, cysteine, aspartic and metalloproteases.[6] Classification of these subclasses is defined by the amino acids present within the enzyme active site that are directly involved in hydrolysis. Proteolytic enzymes perform a diverse range of biological functions, including blood coagulation and fibrinolysis, peptide hormone processing, transportation of secretory proteins across membranes, macromolecular structure assembly, fertilisation and digestion.[7] An imbalance in the normal function of these enzymes can lead to the development of a wide range of disease states.

Serine proteases contain a highly conserved catalytic triad of amino acids, denoted as Ser195, His57 and Asp102.[8] The numbers denote the position of the residue in the polypeptide chain. Scheme 1.5 illustrates the serine protease catalysed hydrolysis of a peptide bond. Nucleophilic attack by the hydroxyl group of Ser195 upon the peptide substrate **1.8** leads to the formation of the tetrahedral intermediate **1.11**. The nucleophilicity of Ser195 is enhanced by the His57 and Asp102 residues, which act as an electron sink and source respectively in a charge-transfer system. The oxyanion of **1.11** is stabilised by hydrogen bonding to NH groups in an active site region termed the "oxyanion hole". Conversion of **1.11** to the acyl-enzyme intermediate **1.12** is accompanied by release

of the *C*-terminal fragment **1.10** into the surrounding milieu. Hydrolysis of **1.12** by an active site water molecule releases the *N*-terminal fragment **1.9**, and the catalytic triad is regenerated.



Scheme 1.5. Serine protease catalysed hydrolysis of a peptide bond.

The highly conserved mechanism of peptide bond hydrolysis by serine proteases does not restrict this class of enzymes to a narrow range of functions in biological systems. This is illustrated by the diversity of physiological roles performed by the representative serine proteases displayed in Table 1.1.[9]

Enzyme	Source	Function	Peptide bond cleaved (i.e. P_1-P_1')
Trypsin	Pancreas	Digestion of proteins	Arg-X or Lys-X
Chymotrypsin	Pancreas	Digestion of proteins	Large hydrophobic-X
Elastase	Pancreas	Digestion of elastin	Ala-X or Gly-X
Thrombin	Blood serum	Blood clotting	Arg-Gly in fibrinogen
Kallikrein	Many tissues and body fluids	Generation of kinins, pain sensing	Arg-X
Acrosomal protease	Acrosome of sperm	Penetration of zona pellucida of ovum	Arg-X or Lys-X
Lysosomal proteases	Animal cells, plant roots	Cell destruction, defence against microbes	

Table 1.1. Examples of serine proteases, showing source, function and site of cleavage.

Proteolytic enzyme cleavage sites are dictated by the chemical nature of the residues adjacent to the site of peptide bond hydrolysis (see right-hand column of Table 1.1). In general, the affinity of an enzyme for a particular substrate or inhibitor is defined by the nature of specificity "pockets" within the active site.[7,10] For example, a pocket of a non-polar nature would rather be occupied by a non-polar amino acid side chain than a polar one. Schechter and Berger devised a nomenclature system[11] to describe the binding between an enzyme and its substrate. Amino acid residues from the *N*-terminus of the substrate cleavage point are named P_1, P_2 etc, and the corresponding subsites of the enzymes are termed S_1, S_2, etc (Figure 1.2). From the *C*-terminus of the substrate cleavage point the residues are named P_1', P_2', etc, while the corresponding subsites are denoted S_1', S_2', etc. It can be seen from Table 1.1 that each serine protease has a specific site of cleavage, *e.g.* chymotrypsin-like enzymes prefer a large hydrophobic residue at P_1. However, interactions are not wholly dictated by the P_1/S_1 contact, and residues as distant as P_{14}' have been determined to influence binding.[12]

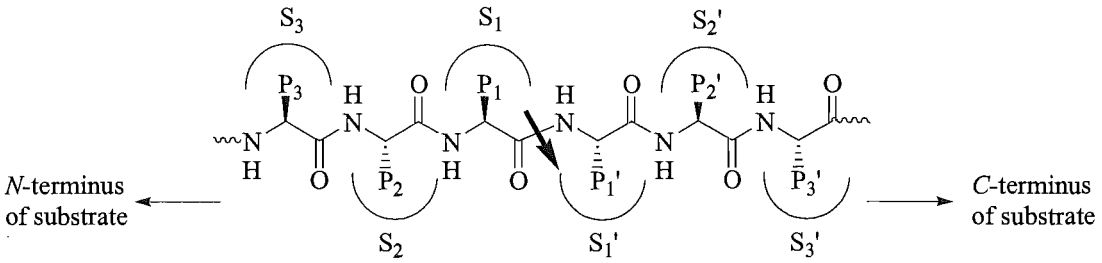


Figure 1.2. Schematic representation of Schechter-Berger nomenclature. The point of cleavage is represented by a bold arrow.

An imbalance in the activity of a particular serine protease can result in an adverse pathological condition. The following serine proteases have been implicated in specific disease states: α-chymotrypsin (digestive disorders); cathepsin G (inflammation); mast cell chymase (inflammation and psoriasis); human leukocyte elastase (acute respiratory distress syndrome, rheumatoid arthritis, atherosclerosis, pulmonary emphysema and cystic fibrosis); and kallikrein (imbalance with the enzyme renin to give renal failure).[13] Control of the over-activity of these enzymes with potent and specific inhibitors is considered a means to develop efficient therapies for these diseases.

One approach to reduce protease activity is to develop enzyme inhibitors. Three binding modes, from which new classes of serine protease inhibitors can be generated, are outlined below. The first is the design of a scaffold where binding to the enzyme is enhanced by the presence of a metal ion relative to the inhibition elicited by the scaffold itself.[14] A second mode of inhibition is derived from a relatively inactive precursor that possesses latent reactivity, which is released in the enzyme active site upon initiation of the enzymes normal catalytic cycle. This mechanism-based or "suicide" inhibition allows for the formation of a covalent bond between the inhibitor and its target enzyme.[15] The third inhibitory mode relies on conformationally restricted molecules that mimic the peptide bond of the natural substrate. The peptide bond isostere is "locked" into the desired shape by a cyclic structure.[16] There is a general focus in these laboratories toward the design and synthesis of α-chymotrypsin inhibitors that utilise one of the binding modes described above. α-Chymotrypsin is an excellent model system for serine protease inhibition due to its well-defined active site structure[17] and substrate specificity.[7a,10,18] Inhibition of α-

chymotrypsin is also regarded as an important step in the development of inhibitors for chymotrypsin-like enzymes such as cathepsin G, mast cell chymase and human leukocyte elastase.[13c]

What follows in the remainder of Section 1.2 is a discussion of serine protease inhibition by two methods mentioned in the previous paragraph. In both cases, compounds with latent reactivity are utilised to elicit a specific interaction with the biological target. Specifically, the metal-enhanced binding of selected scaffolds to serine proteases, and the application of mechanism-based inhibition towards α-chymotrypsin, is addressed.

1.2.1 Enzyme inhibitors with metal-ion enhanced activity

One issue of concern in the discipline of medicinal chemistry is that compromised bioavailability can arise in enzyme inhibitors that possess complex peptidomimetic structures and a high molecular weight.[14] An improvement in bioavailability can be obtained by decreasing the peptidic character of inhibitors (*i.e.* fewer amide bonds, achiral) and of a molecular weight lower than 500 Da.[14] The development of structures that incorporate the aforementioned structural features and exhibit high affinity and selectivity for a particular target is of ongoing concern.

Katz, *et al*[19] effected inhibition of the representative serine proteases trypsin, tryptase and thrombin with benzimidazole-based compounds of type **1.13**. A selection of the structures (**1.13a-e**) is illustrated in Figure 1.3. Note that these compounds possessed the desired features of a nonpeptidic structure and low molecular weight.

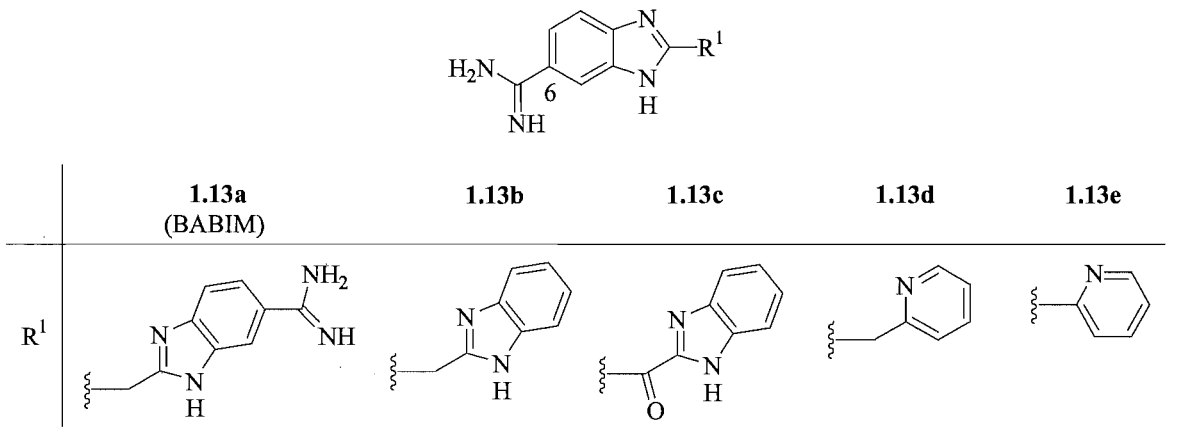


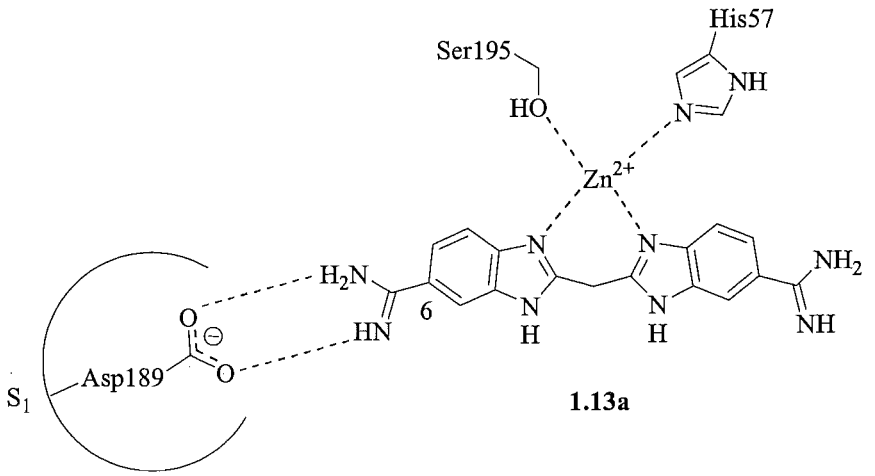
Figure 1.3. Serine protease inhibitors **1.13a-e**.

The inhibition data for **1.13a-e** against a range of serine proteases is presented in Table 1.2. Inhibition constant (K_i) values are a measure of the dissociation constant for the enzyme-inhibitor complex[20] – the lower the number, the greater the level of inhibition. Addition of Zn^{2+} at concentrations as low as 100 nM gave a significant increase in the potency of these compounds. The increase was quantified as the "Zn^{2+} factor" (right-hand column of Table 1.2), which was given by the K_i value for a compound in the presence of EDTA divided by the K_i observed for the same compound when Zn^{2+} was present. EDTA was added to determine the activity of the compounds when no metal ions were present. An example is inhibitor **1.13a** (BABIM), which inhibited trypsin in the presence of EDTA with K_i = 18.8 μM. Addition of Zn^{2+} lowered the K_i value to 90 nM, *i.e.* an increase in activity by a factor of 210. The closely related analogue **1.13c** had a remarkable 17,000-fold increase in inhibitory activity against trypsin, with K_i = 87.5 μM in the presence of EDTA lowered to K_i = 5.2 nM when Zn^{2+} is present. The activity towards serine proteases of **1.13a-e** is modulated upon Zn^{2+} binding, *i.e.* a change in physical properties effects greater inhibition.

No.	Human protease	Inhibition (K_i, μM)		Zn^{2+} factor, *i.e.* (1)/(2)
		+ EDTA(1)	+ Zn^{2+}(2)	
1.13a	Trypsin	18.8	0.09	210
	Tryptase	2.5	0.005	500
	Thrombin	3.7	0.023	160
	Chymotrypsin	710	120	6
	Chymase	>1,000	>1,000	-
1.13b	Trypsin	182	0.0235	7,700
	Tryptase	13.5	0.0695	190
	Thrombin	2.31	0.0058	410
1.13c	Trypsin	87.5	0.0052	17,000
	Tryptase	5.72	0.05	110
	Thrombin	>1,000	0.101	>9,900
1.13d	Trypsin	>1,000	136	>7
	Tryptase	358	0.31	1,200
	Thrombin	>1,000	10.5	>95
1.13e	Trypsin	101	20.9	5
	Tryptase	19.3	1.6	12
	Thrombin	81.5	0.721	110

Table 1.2. Inhibition data for **1.13a-e** against a range of serine proteases in the presence of EDTA or Zn^{2+}.

The mode of binding for **1.13a** to trypsin was determined by X-ray crystallography, and it revealed that the enzyme was inhibited by the formation of a ternary complex between active site residues, a Zn^{2+} ion and the inhibitor. The nature of this complex is displayed in Figure 1.4, whereby the Zn^{2+} ion was tetrahedrally coordinated by one nitrogen from each of the two benzimidazole ring systems of **1.13a**, a nitrogen from the imidazole side chain of His57, and the hydroxyl group oxygen of Ser195.



1.13a

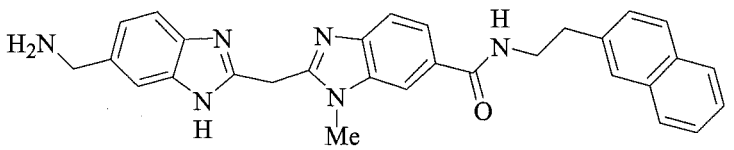
Figure 1.4. Structure of the ternary complex formed between BABIM (**1.13a**), Zn^{2+} and the Ser195 and His57 residues of trypsin. The interaction between Asp189 at the base of the S_1 subsite of trypsin and the amidine group of **1.13a** is also illustrated.

Specificity for inhibitors of type **1.13** against trypsin-like enzymes was conferred by the amidine group at C6. The amidine functionality forms a salt bridge with residue Asp189 at the base of the S_1 subsite, as illustrated in Figure 1.4. Trypsin-like enzymes prefer basic residues such as lysine or arginine at the P_1 position of its natural substrate,[10] which suggested that an interaction between Asp189 and the amidine group would be favoured. As demonstrated by the K_i values and Zn^{2+} factors obtained, **1.13a** was not an effective inhibitor of chymotrypsin and chymase. These enzymes have a preference for substrates with an aromatic group at the P_1 position,[10] therefore the amidine group of **1.13a** would not favourably interact with the S_1 subsite of chymotrypsin or chymase.

The choice of metal ion was discovered to be important for the enhancement of tryptase inhibition by **1.13a**, with Zn^{2+} determined as the optimal metal ion.[21] Ca^{2+}, Cu^{2+}, Mg^{2+} and Ni^{2+} elicited no additional inhibition compared to when EDTA was present, and only slight enhancements (less than 10-fold) were observed with Cd^{2+}, Co^{2+} and Mn^{2+}.

Further publications have diversified the scaffolds upon which the metal ion-enhanced inhibition of serine protease can be based. Janc, *et al*[21] synthesised a series of compounds based on the bis(benzimidazole)methane scaffold utilised by Katz, *et al.*[19] One such

inhibitor (**1.14**, Figure 1.5) displayed a remarkable enhancement of 97,000-fold for tryptase in the presence of Zn^{2+}, compared to when ternary complex formation was prevented by the addition of EDTA. In addition, a high degree of selectivity for tryptase over trypsin and thrombin was exhibited.



1.14

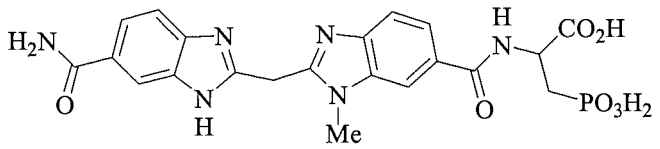
Tryptase K_i (EDTA) = 6.6 μM
K_i (Zn^{2+}) = 0.068 nM
Zn^{2+} factor = 97,000

Trypsin K_i (Zn^{2+}) = 670 nM

Thrombin K_i (Zn^{2+}) = >1 mM

Figure 1.5. Structure of **1.14** and its inhibition data against tryptase, trypsin and thrombin.

Compounds **1.15**[22] and **1.16**[23] were synthesised as part of a study towards inhibition of hepatitis C virus NS3 serine protease (Figure 1.6). NS3 protease is a chymotrypsin-like viral enzyme involved in polyprotein processing, an integral step towards the production of viable viral particles.[24] The inhibition data for **1.15** was expressed as an IC_{50} value - this is the concentration of inhibitor required for half-maximal inhibition of the target enzyme.[25] Inhibitory activity of **1.15** was improved by conversion of the amide functionality on the left-hand side of the molecule to a phosphonoalanine, replacement of the methylene bridge by a difluoromethylene group, and elaboration of the right-hand phosphonoalanine into a more complex phosphonate derivative to afford **1.16**. These findings showed that the phenomenon of zinc-enhanced inhibition can be extended to non-mammalian serine proteases.

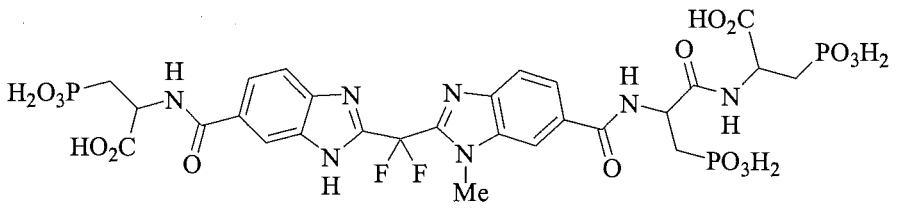


1.15

IC_{50} (EDTA) = 167 μM

IC_{50} (Zn^{2+}) = 0.20 μM

Zn^{2+} factor = 835



1.16

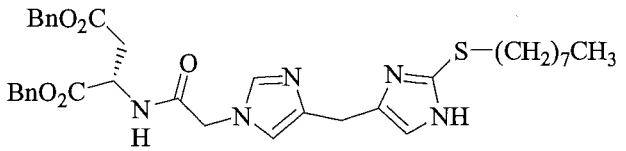
K_i (EDTA) = 1 μM

K_i (Zn^{2+}) = 27 nM

Zn^{2+} factor = 37

Figure 1.6. Structure of **1.15** and **1.16**, and the data for inhibition against hepatitis C virus NS3 serine protease.

Metal ion-mediated inhibition of viral enzymes has also been applied to the human cytomegalovirus serine protease.[26] The bis(benzimidazole)methane template of an inhibitor such as **1.13a** was considered too large to be accommodated in the enzyme active site. Therefore, inhibitor **1.17** was based on a less sterically demanding bis(imidazole)methane motif (Figure 1.7). The 4,4'-bis(imidazole)methane system was chosen over the 2,2' isomer to minimize the potential of oxidation at the methylene linker. However, **1.17** was only a moderate inhibitor of the target viral protease, and it exhibited minimal enhancement of activity in the presence of Zn^{2+}. The other bis(imidazole)methane-based analogues prepared in this study had little or no activity in the absence of free Zn^{2+}, but on the addition of the metal ion inhibition was observed. This was consistent with the formation of a ternary complex to aid inhibition.



1.17

IC_{50} (EDTA) = 15 μM

IC_{50} (Zn^{2+}) = 5 μM

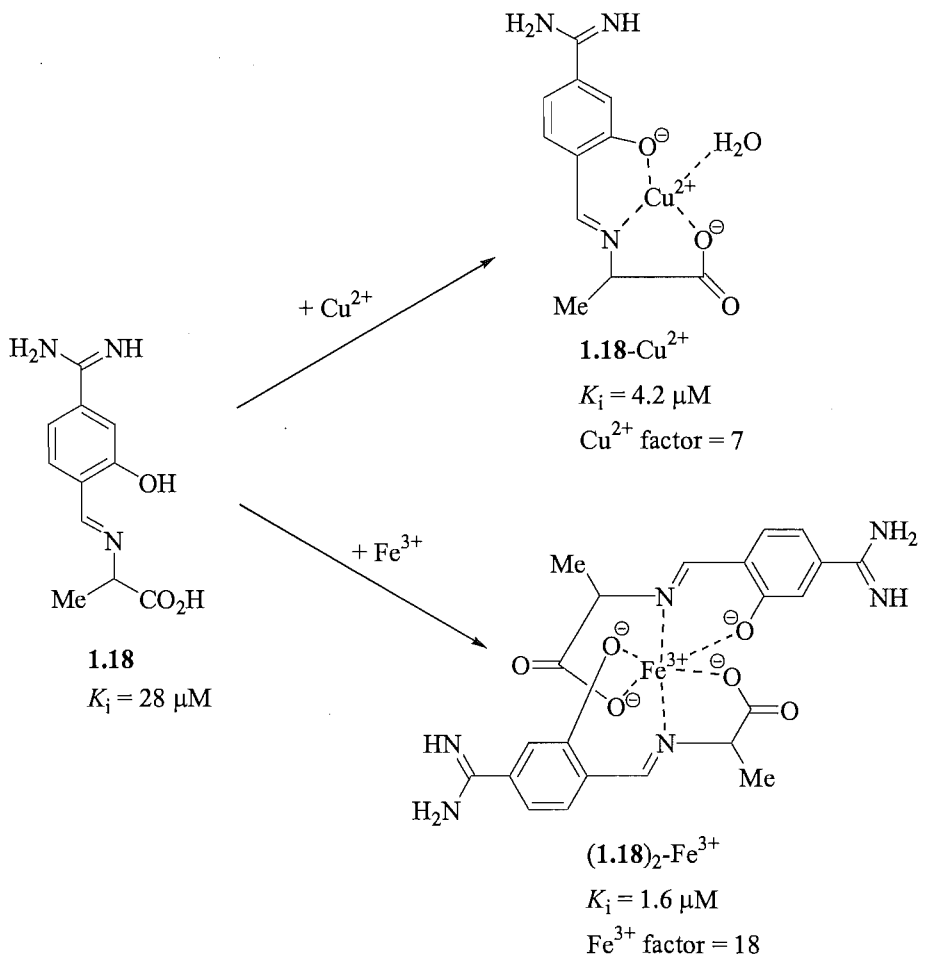
Zn^{2+} factor = 3

Figure 1.7. Structure of **1.17** and the data for inhibition against human cytomegalovirus serine protease.

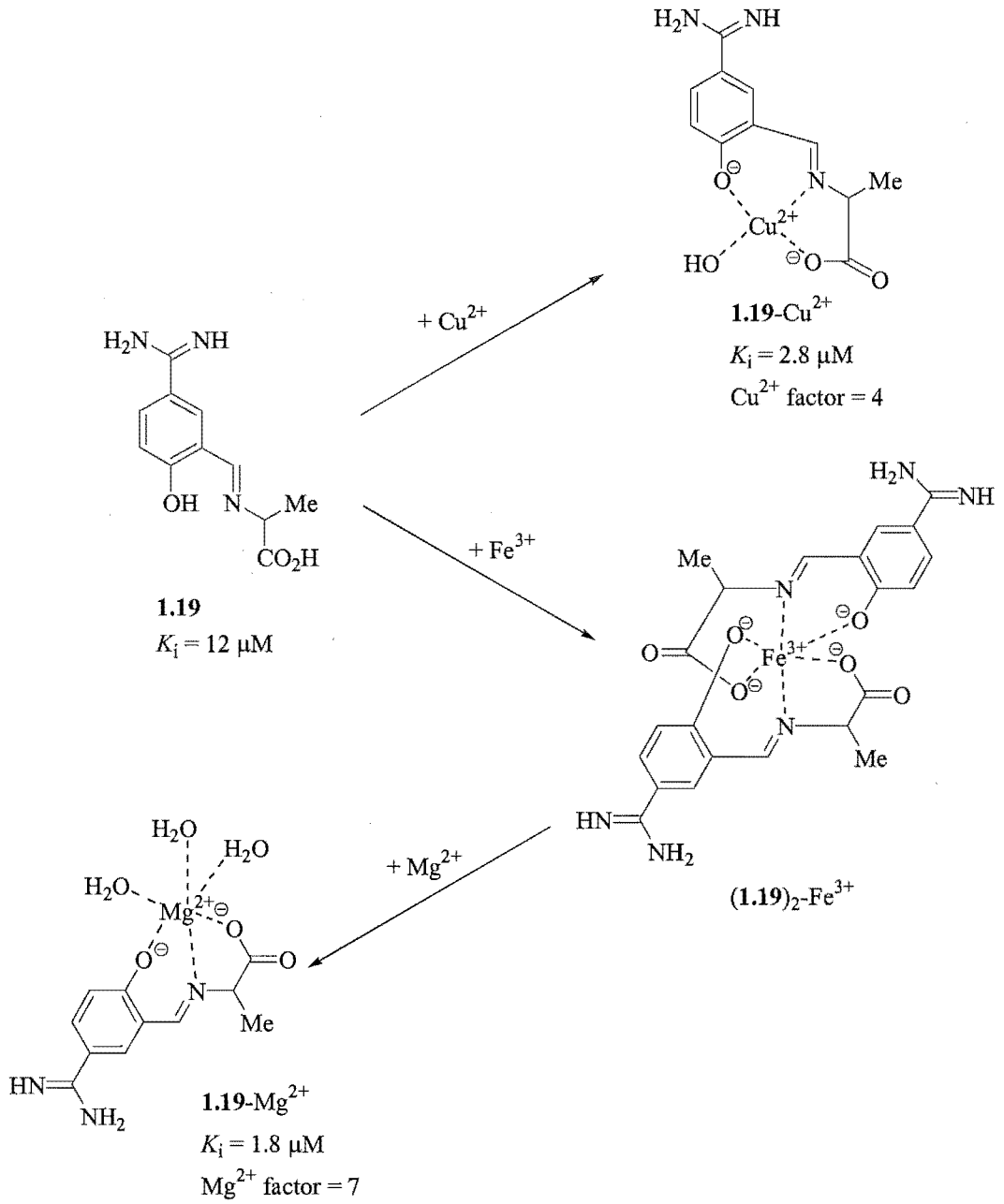
The enhanced inhibition of bovine β-trypsin has been achieved in a metal ion-mediated manner.[27] Benzamidines **1.18** and **1.19** were moderate inhibitors of this enzyme (inhibition data displayed in Schemes 1.6a and 1.6b). Upon the addition of Cu^{2+} or Fe^{3+} ions, the activity of **1.18** and **1.19** was enhanced to some degree. The metal ions differed in that Cu^{2+} formed a 1:1 complex with either benzamidine, whereas Fe^{3+} existed in 1:2 complexes with either **1.18** or **1.19**. Activity against bovine β-trypsin of **1.18** and **1.19** was enhanced to a greater degree by Fe^{3+} in comparison to Cu^{2+}. The four complexes interacted differently with the active site of bovine β-trypsin as determined by X-ray crystallography. All complexes formed a salt bridge between the amidine moiety of **1.18** and **1.19** to Asp189, Ser190 and Gly219. However, **1.19**-Cu^{2+} was the only complex where an active site residue from the catalytic triad (His57) acted as a ligand to the metal ion. The crystal structure of (**1.19**)$_2$-Fe^{3+}, in the active site of bovine β-trypsin, revealed two surprising alterations to its structure. One of the two benzamidine ligands of complex (**1.19**)$_2$-Fe^{3+} was lost (probably to avoid unfavourable interactions with active site residues), and the Fe^{3+} ion was replaced by Mg^{2+}. The new complex **1.19**-Mg^{2+} was indirectly attached to two catalytic triad residues (Ser195 and His57) through a water molecule. Complex (**1.18**)$_2$-Fe^{3+} contacted Gln192 via one of its carboxylate ligands, whereas **1.18**-Cu^{2+} was shielded from any contact with active site residues other than those mediated by its amidine group. For all cases except that of **1.19**-Cu^{2+}, the mode of binding was such that no ligand site of the metal ion was occupied by a catalytic triad residue. This differed to the ternary complex formed by **1.13a**, Zn^{2+} and the enzyme, *i.e.* Ser195 and

His57 acted as ligands towards Zn^{2+}. However, these binding modes revealed alternative means by which inhibition of the target enzyme can be achieved in a conceptually similar manner to that of **1.13a**, *i.e.* the presence of a metal ion increased the potency of an inhibitor.

(a)

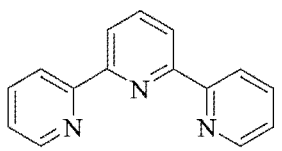


(b)

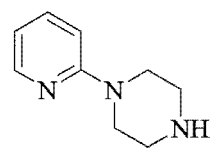


Scheme 1.6a and 1.6b. Complex formation between Cu^{2+} and the Schiff bases **1.18** (Scheme 1.6a) and **1.19** (Scheme 1.6b) gave **1.18**-Cu^{2+} and **1.19**-Cu^{2+} respectively. Complex formation between Fe^{3+} and the Schiff bases **1.18** (Scheme 1.6a) and **1.19** (Scheme 1.6b) gave (**1.18**)$_2$-Fe^{3+} and (**1.19**)$_2$-Fe^{3+} respectively. (**1.19**)$_2$-Fe^{3+} was converted to **1.19**-Mg^{2+} in the active site of bovine β-trypsin. Inhibition data for all compounds/ complexes against the enzyme is presented.

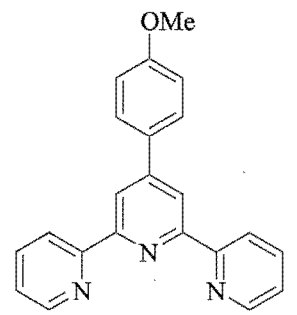
Blum *et al* developed an "on-off" switch for serine protease inhibition.[28] In an uncomplexed form, none of the tridentate ligands **1.20-1.22** presented in Figure 1.8 inhibited Kex2, a yeast serine protease. Kex2 is involved in the processing of peptide precursors into biologically active proteins that are secreted from the cell.[29] Inhibition of Kex2 is viewed as an excellent model system towards the inhibition of furin, a ubiquitous mammalian processing enzyme, as both enzymes have sequence homology and similar physiological roles. Potential antiviral therapies could arise from the selective inhibition of furin, as this enzyme inadvertently activates the envelope glycoproteins of many viruses to enable the uptake of viral particles by host cells.[30] Upon addition of Cu^{2+} ions to separate samples of **1.20-1.22**, the newly formed complexes displayed activity in the micromolar range against Kex2. In contrast, attempts to elicit Kex2 inhibition by the complexation of **1.20-1.22** with Zn^{2+} did not result in enhanced inhibitory activity.



1.20 $IC_{50} = 20\ \mu M$



1.21 $IC_{50} = 75\ \mu M$



1.22 $IC_{50} = 10\ \mu M$

Figure 1.8. Kex2 inhibitors **1.20-1.22**, and the inhibition data for each compound in the presence of Cu^{2+}.

Metal ions are not the only entities that can enhance serine protease inhibition. Williams, *et al*[31] synthesised a series of aryl benzamidines as proposed bovine β-trypsin inhibitors. At pH 6.0, compound **1.23** demonstrated an enhancement of activity by a factor of 5.3 as a result of the formation of a ternary complex between inhibitor, sulfate (SO_4^{2-}) ion and enzyme (Figure 1.9). At pH 7.5 this enhancement was considerably diminished.

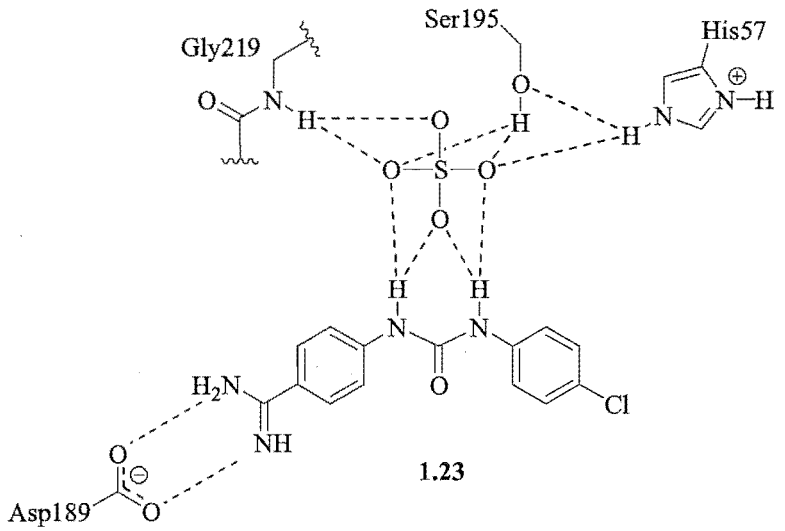


Figure 1.9. Ternary complex formed between **1.23**, SO_4^{2-} and bovine β-trypsin, in addition to the inhibition data at pH 6.0 and 7.5.

Compounds that bind a metal ion to elicit a greater affinity for biological targets other than serine proteases have also been documented. The following examples illustrate that specific points in the HIV life cycle can be disrupted by metal-organic complexes. Xylyl-bicyclam **1.24** (Figure 1.10),[32] upon the coordination of $Zn(OAc)_2$, adopted a conformation that elicited a strong interaction with the CXCR4 receptor, which is employed by HIV for membrane fusion and entry into the cell.[33] The CXCR4 antagonist activity of **1.24** toward HIV binding increases by 7-, 36-, and 50-fold upon incorporation of Cu^{2+}, Zn^{2+}, and Ni^{2+} respectively.[34] Pseudopeptide **1.25** (Figure 1.10) displayed an 7-fold increase in the presence of Zn^{2+} in its ability to inhibit HIV-1 protease dimerisation.[35] Prevention of dimerisation halts the processing of viral polyproteins. Several non-peptidic metal-organic complexes also inhibit HIV-1 protease. Examples include the porphyrin-Cu^{2+} complex **1.26**,[36] the bathocuproine-Cu^{+} complex **1.27**,[37] bis-(2-pyridylcarbonyl)-amido-Cu^{2+} complex **1.28**,[38] and the benzamide-Cu^{2+} complex **1.29**[39] (Figure 1.10).

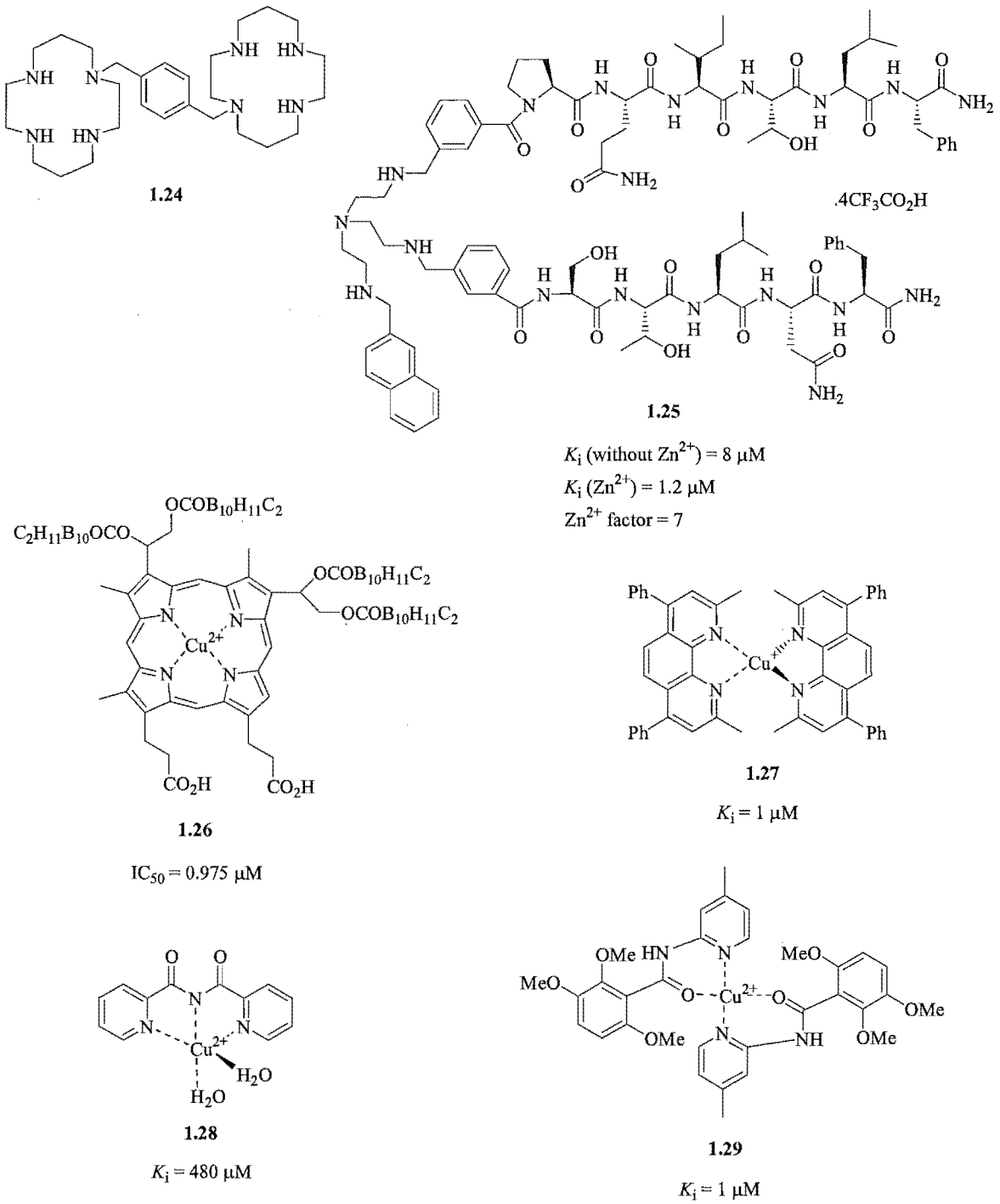
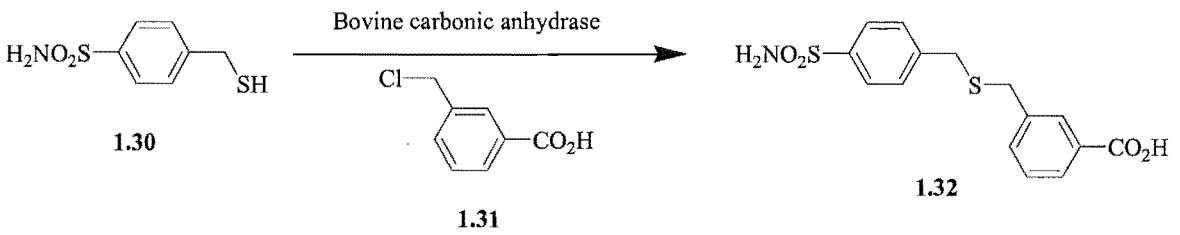


Figure 1.10. Anti-HIV agents **1.24-1.29**. Inhibition data is included for **1.25-1.29**.

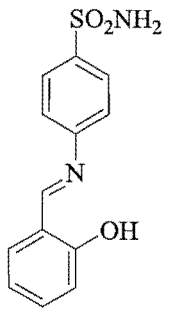
Examples **1.26-1.29** illustrate how a pre-formed complex can be employed to elicit enzyme inhibition. Conversely, inhibitors have been designed to take advantage of the metal ions

already present in the active site, *i.e.* metalloenzyme-specific inhibition. The inhibition of BoNT/B-LC (botulinum neurotoxin serotype B, light chain) with BABIM (**1.13a**, see Figure 1.3) gave K_i = 1.6 μM.[40] BoNT/B-LC is a metalloenzyme produced by the bacteria *Clostridium botulinum* that cleaves host neuropeptides, producing the paralytic symptoms of botulism.[41] Subsequent analysis revealed that a ternary complex was initially formed between **1.13a**, the enzyme and its catalytic Zn^{2+} ion, and the metal ion was then slowly displaced from the active site.[42] Another Zn^{2+} metalloenzyme, bovine carbonic anhydrase, preassociated with the thiol **1.30** (Scheme 1.7) via co-ordination to Zn^{2+} in the active site.[43] Addition of an alkyl chloride (the optimal example **1.31** is displayed in Scheme 1.7) gave **1.32**, which inhibited bovine carbonic anhydrase with K_i = 59 μM.



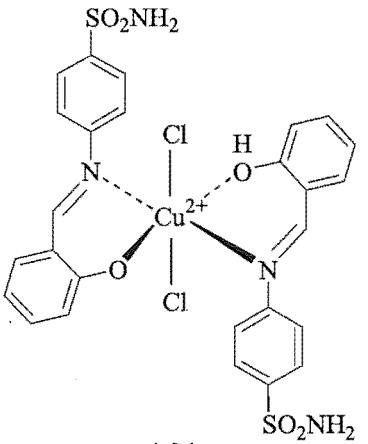
Scheme 1.7: Inhibition of bovine carbonic anhydrase by formation of **1.32** in the active site.

Three isozymes of carbonic anhydrase (CA I, CA II and CA IV) were inhibited by the Schiff base **1.33** (Figure 1.11).[44] Increased inhibition was observed with Co^{2+}, Ni^{2+} and Cu^{2+}, and the Cu^{2+} complex **1.34** is displayed in Figure 1.11. This is an example of a pre-formed complex inhibiting a metalloenzyme, as opposed to the previous examples of metalloenzyme inhibitors that were purely organic compounds.



1.33

K_i (CA I) = 28 μM
K_i (CA II) = 300 nM
K_i (CA IV) = 3 μM

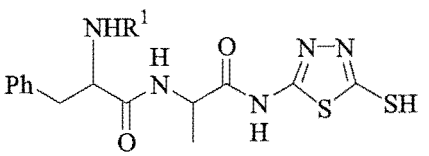


1.34

K_i (CA I) = 210 nM
Cu^{2+} factor = 133
K_i (CA II) = 13 nM
Cu^{2+} factor = 23
K_i (CA IV) = 83 nM
Cu^{2+} factor = 36

Figure 1.11. Inhibitory data for the Schiff base **1.33** and its Cu^{2+} complex **1.34** against CA I, CA II and CA IV

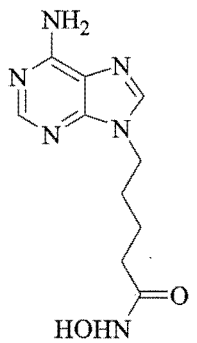
Other zinc proteases targeted include the matrix metalloproteases (MMPs) and the bacterial proteases (BPs).[45] These enzymes are of medicinal interest due to involvement in cancer and arthritis (MMPs), or as targets for potential antibacterial agents (BPs). Inhibition of several representative MMP and BP examples, based on the inhibitor scaffolds **1.35a-b** (Figure 1.12), was recorded into the low micromolar range.



1.35a $R^1 = SO_2R^2$
1.35b $R^1 = CONHSO_2R^2$

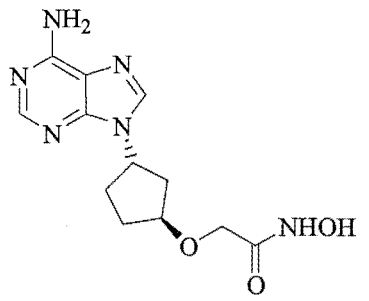
Figure 1.12. Structure of MMP and BP inhibitor scaffolds **1.35a-b**.

Inhibition of adenylyl cyclase (AC) isoform V has been achieved with a series of inhibitors that complexed the two Mg^{2+} ions present in the active site via a hydroxamate moiety.[46] AC catalyses the conversion of adenosine triphosphate to the important cell signaling molecule cyclic adenosine monophosphate.[47] Selective inhibition of the nine isoforms of AC would give therapeutic agents with differing effects on metabolic activity. Figure 1.13 illustrates the optimal AC isoform V inhibitors for two series of analogues. One series possessed an acyclic linker between the adenine moiety and the hydroxamate (optimal inhibitor **1.36**), and the other series a cyclic linker (optimal inhibitor **1.37**).



1.36

$IC_{50} = 7.6\ \mu M$



1.37

$IC_{50} = 10.8\ \mu M$

Figure 1.13. Structure and inhibition data for the adenylyl cyclase inhibitors **1.36** and **1.37**.

Other biological targets studied include the prostate-specific antigen (PSA), whereby Zn^{2+} increased the affinity of cysteine-containing peptides for PSA.[48] These peptides have potential for the identification of PSA variants and for the imaging and targeting of prostate tumours. A mutation to introduce a metal-binding cysteine into seven transmembrane receptor proteins has resulted in its activation by small hydrophobic chelators complexed with Zn^{2+}, or by Zn^{2+} or Cu^{2+} ions themselves.[49]

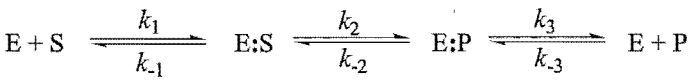
In summary, a wide variety of compounds have displayed a metal-mediated enhancement of activity toward certain biological targets. Serine proteases exhibit a tendency to be inhibited in this fashion. Inhibition of this class of enzymes can be effected by other

methods, and the following section addresses the concept of mechanism-based inhibition and its application to serine proteases.

1.2.2 Mechanism-based enzyme inhibitors

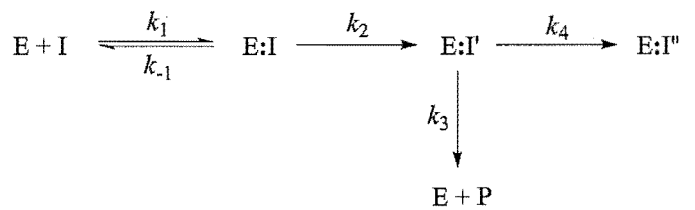
A diverse range of compounds, both natural and synthetic, have demonstrated inhibitory activity toward enzymes. Classification of these inhibitors results in two general categories that describe the mode of binding. The first group are termed reversible inhibitors, and these compounds merely reside in the active site of the enzyme, with no covalent linkage formed between the enzyme and inhibitor.[15] The second group irreversibly inactivates the enzyme as a result of covalent derivatisation by a reactive inhibitor species.[15] A subset of the irreversible inhibitors are the mechanism-based or "suicide" inhibitors.[50] These compounds are relatively unreactive molecules that possess structural similarity to the substrate, which endows specificity upon the inhibitor towards the target enzyme. Docking of the inhibitor into the enzyme active site releases the compounds latent reactivity upon initiation of the enzymes catalytic cycle. A covalent linkage is subsequently formed between an active site residue and the reactive inhibitor species, preventing further enzymatic activity, *i.e.* inhibition is effected by the modulation of the compounds chemical properties. A higher degree of enzyme specificity for a particular enzyme can be obtained by mechanism-based inhibitors over conventional active site inactivators. This observation is expected as the suicide inhibitors possess similarity to the substrate, in addition to activity being dependent on the catalytic mechanism of the target enzyme.

The differences between the catalytic steps involved when an enzyme reacts with its normal substrate or a mechanism-based inhibitor are outlined below. For the reaction of an enzyme (E, Scheme 1.8) with its substrate (S),[51] an enzyme-substrate complex (E:S) is formed. This is converted to the enzyme-product complex (E:P), with subsequent release of the product (P). All steps are reversible.



Scheme 1.8. Reaction of an enzyme (E) and its substrate (S) to give product (P).

In contrast, reaction between an enzyme (E) and a mechanism-based inhibitor (I) initially forms the enzyme-inhibitor complex (E:I, Scheme 1.9). The latent reactivity of the inhibitor is then released by enzyme catalysis to form the enzyme-activated inhibitor complex (E:I'). This complex cannot undergo the reverse reaction to reform E:I, and instead either reaction k_3 or k_4 occurs. P is the product released by cleavage of E:I' (reaction k_3), and E:I'' is the enzyme-inhibitor adduct resulting from the formation of a covalent bond between the two species (reaction k_4).



Scheme 1.9. Reaction of an enzyme (E) and an inhibitor (I) to give the enzyme-inhibitor adduct E:I''.

The partition ratio, which is equal to k_3/k_4, is the ratio of the number of latent inactivator molecules (E:I') converted to product (P) relative to the turnover of E:I' leading to enzyme inactivation. This ratio is a measure of the inhibitors efficiency towards enzyme deactivation.

Mechanism-based inhibition has been applied to a wide range of biological targets, utilising a diverse array of inhibitor scaffolds.[51,52] Inhibition of serine proteases has been achieved through the rational design and synthesis of mechanism-based inhibitors.[53] In particular, α-chymotrypsin, the target enzyme of these laboratories, has been inactivated in a mechanism-based fashion by a diverse range of heterocyclic systems. Some representative examples include the succinimide **1.38**,[13c] coumarin **1.39**[13d] and isocoumarin

1.40,[54] pyrone **1.41**,[55] benzoxazinone **1.42**,[56] isatoic anhydride **1.43**,[57] and the haloenol lactones **1.44**[58] and **1.45**[59] (Figure 1.14).

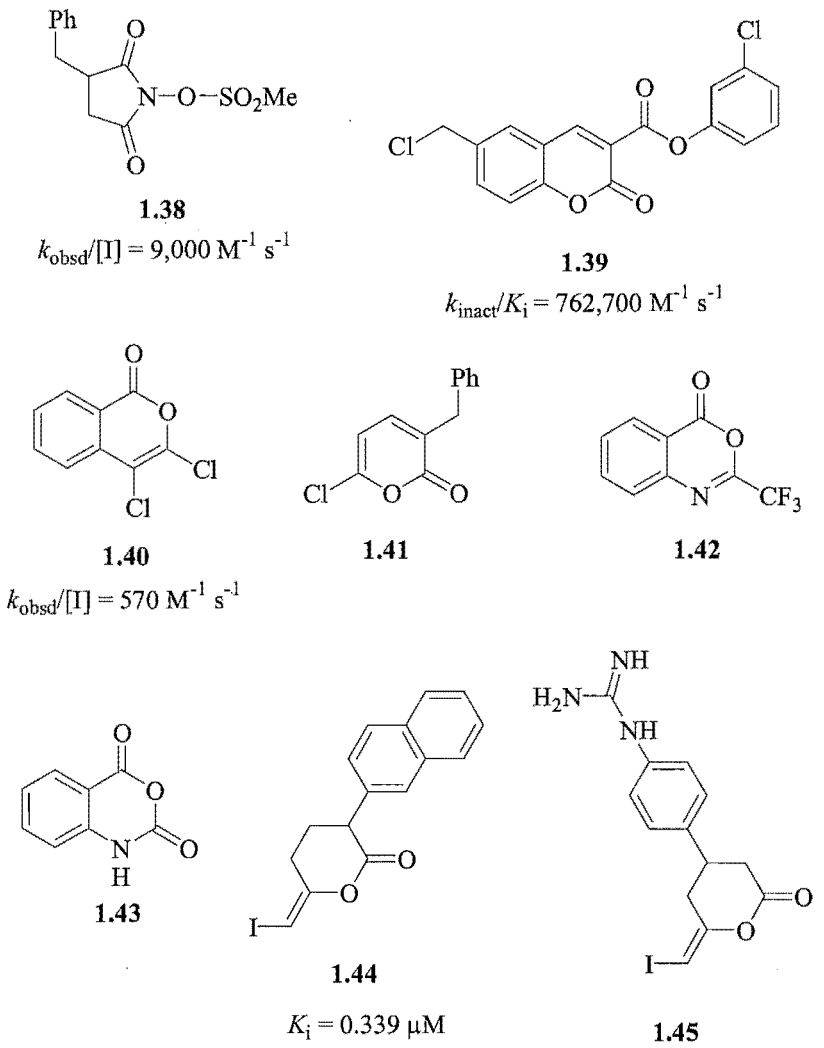
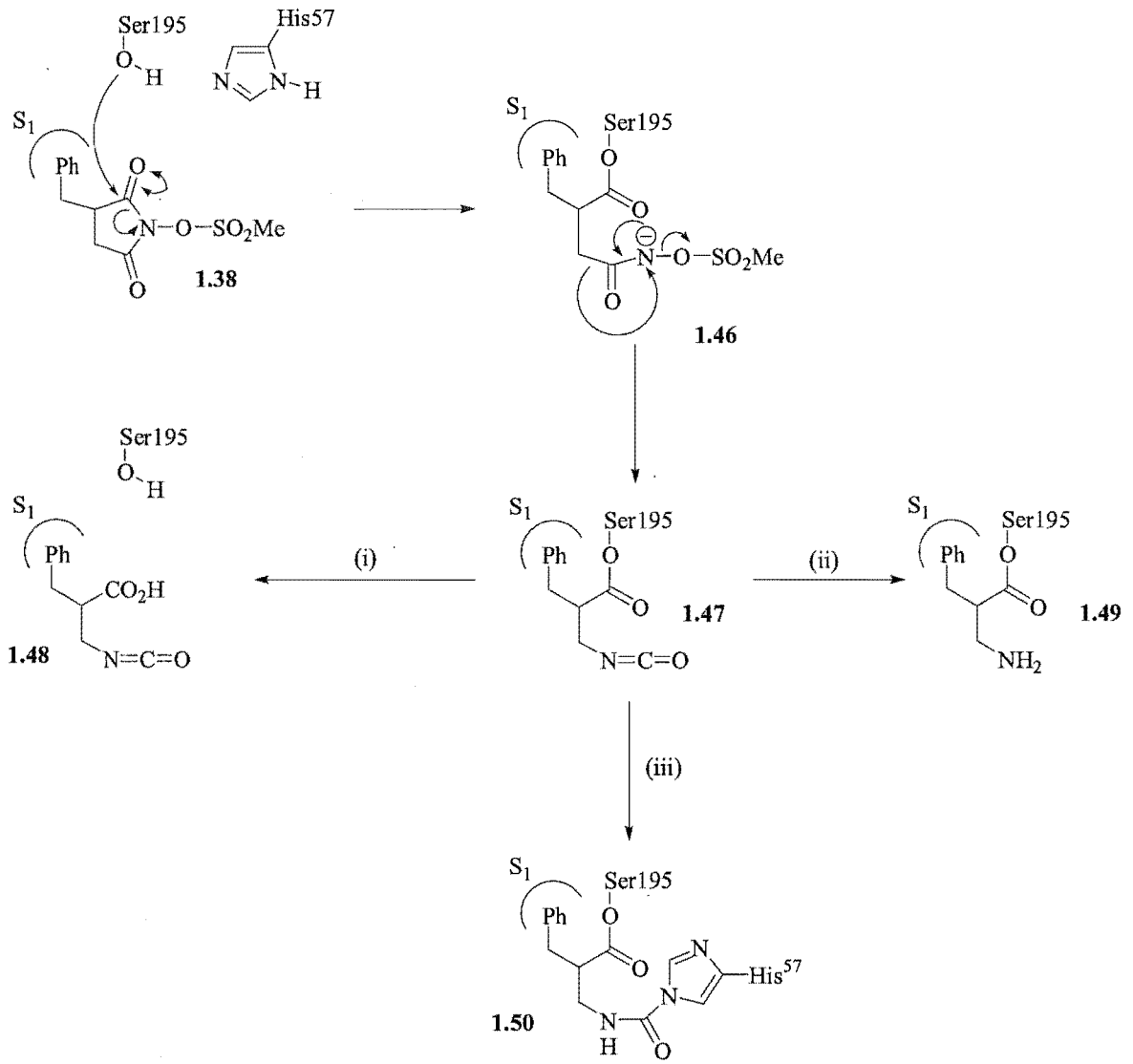


Figure 1.14. Structures of the mechanism-based α-chymotrypsin inhibitors **1.38-1.45**. Inhibition data is given where appropriate.

The reaction sequence determined for succinimide **1.38** upon its arrival in the active site of α-chymotrypsin[13c] serves as a representative example of mechanism-based enzyme inhibition. Nucleophilic attack by the hydroxyl group of Ser195 on **1.38** gave **1.46** (Scheme 1.10), which instantaneously collapsed via a Lossen rearrangement to afford the enzyme-bound isocyanate **1.47.** This highly reactive species was postulated to undergo three possible pathways: (i) hydrolysis of **1.47** to release the isocyanate derivative **1.48** into

the surrounding milieu; (ii) hydrolysis of the isocyanate functionality to afford the mono-linked enzyme adduct **1.49**; (iii) reaction of His57 with the isocyanate to give **1.50**, whereby the inhibitor is covalently attached to the enzyme at two sites.



Scheme 1.10. Reaction of **1.38** in the active site of α-chymotrypsin.

Compounds that possess latent reactivity have applications in medicine other than enzyme inhibition. Section 1.3 is a discussion of the alkylation of DNA by reactive species as a form of cancer chemotherapy.

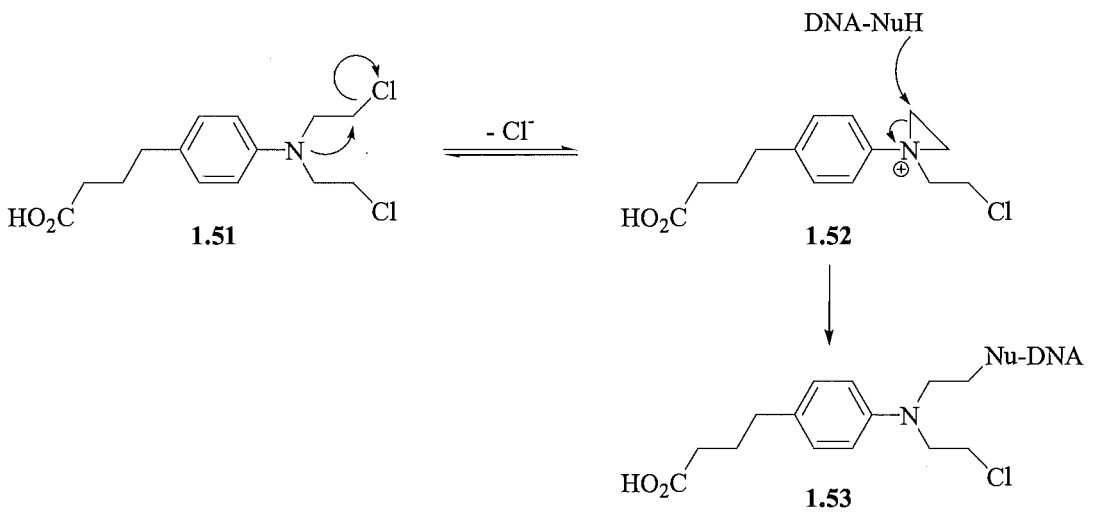
1.3 ANTITUMOUR AGENTS THAT ALKYLATE DNA

Cancer is a general term for more than 100 diseases characterised by the uncontrolled, abnormal growth of cells in the body.[60] Cancer cells cause harm within an organism through the deprivation of nourishment and space to normal cells; formation of a mass (tumour) which can invade and destroy healthy tissue; and by the spread (metastasis) of cancerous cells to other parts of the body via the bloodstream or lymphatic system.

Integral to the continued growth of a tumour is cell replication, which is itself dependent on the action of DNA replication enzymes.[61] Essential to the process of DNA replication are the topoisomerase enzymes, which relieve the substantial conformational strain that accumulates within the genome during replication. Topoisomerase action occurs in two steps, with the first being introduction of strand breaks into the genetic material. Subsequent religation yields a less strained conformation.[62] The topoisomerases are separated into two classes, type I and type II, with classification dependent on the exact mode of action.[63] These enzymes, particularly the type II class, are the target of many antitumour agents.[64] DNA topoisomerase type II inhibitors have an unusual mode of action for an enzyme-targeted agent, as the enzymes normal catalytic function is not inhibited. Instead, the steady-state concentration of the complexes formed between the enzyme and the cleaved DNA is increased, which results in a greater number of aberrations in the genetic material that are lethal to the cell.[65] The levels of topoisomerase types I and II are generally elevated in rapidly dividing cells,[66] so the effect of agents directed at these enzymes is typically more specific for tumour cells. The design of agents specific for DNA topoisomerase type II is an attractive therapeutic option for the treatment of cancer.

One method by which the action of topoisomerase II can be disrupted is by alkylation of the genetic material. A biological alkylating agent is a compound which, under physiological conditions, can replace a hydrogen atom present on a biomolecule with an alkyl group. Many useful clinical agents for the chemotherapeutic treatment of cancer are compounds of this type.[67] The chemical mechanisms by which these compounds alkylate DNA are diverse, and in general the most effective agents are those that are bifunctional

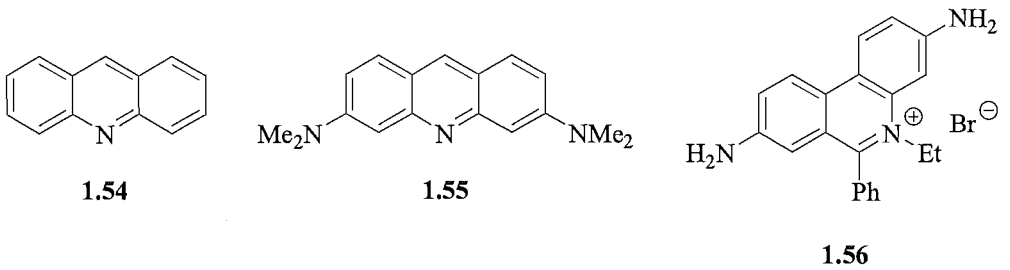
and can crosslink the genetic material, with the N7 position of guanine as the major nucleophilic site in DNA.[68] Chlorambucil (**1.51**),[69] an example of the nitrogen mustard class of alkylating agents, is shown in Scheme 1.11. Alkylation of the genetic material is reliant on the latent reactivity possessed by **1.51**, which is released upon formation of the aziridinium ion **1.52**. Subsequent nucleophilic attack by a DNA nucleophile affords adduct **1.53**, *i.e.* alkylation is dependent on the modulation of the chemical properties of **1.51**. A second electrophilic alkyl halide on **1.53** allows for another alkylation event to occur by an analogous process, *i.e.* release of latent reactivity by formation of an aziridinium ion. This results in a crosslink in the genome which prevents complete DNA replication, and hence cell death occurs.



Scheme 1.11. Chlorambucil (**1.51**) and its reaction with a nucleophilic site in DNA (represented as DNA-NuH) to give adduct **1.53**.

Whilst compounds such as **1.51** are effective antitumour agents, one shortcoming displayed by the simple nitrogen mustards is a lack of selectivity for DNA. Increased affinity for the genetic material is desirable in the design of anticancer compounds, in order to reduce the likelihood that other biological entities present in the cell will interact with the therapeutic agent. As a result, deleterious side effects are lessened in the organism to which the agent is administered. The ability to insert between the pairs of complementary bases in the double helix is one method by which specificity for DNA can be endowed upon a putative antitumour agent. Compounds of this type require a hydrophobic planar

structure to interact favourably with the physical and chemical properties present between the base pairs. The desired specificity for DNA is displayed by a class of compounds known as intercalators, which possess the necessary planar aromatic structure. Three simple intercalators are presented in Figure 1.15 – acridine (**1.54**), acridine orange (**1.55**)[70] and ethidium bromide (**1.56**).[71] Strong mutagenic and carcinogenic properties were displayed by **1.54-1.56** as a result of the high affinity for DNA possessed by these compounds.



1.54 **1.55** **1.56**

Figure 1.15. Structure of the intercalators acridine (**1.54**), acridine orange (**1.55**) and ethidium bromide (**1.56**).

The interaction of ethidium bromide (**1.56**) with DNA is schematically represented in Figure 1.16. The intercalator, represented as a black rectangle, is interspersed throughout the genome. Formation of the intercalator-DNA complex requires separation of adjacent base pairs by approximately 3.4 Å to allow the planar species to enter the genetic material.[72] The unimpeded progress of the replication enzymes along the genome is essential for complete DNA replication, so any obstacle will be deleterious to this process. It has become clear that the primary mode of cytotoxicity of many clinically useful intercalating agents involves inhibition of the religation step of topoisomerase II.[73]

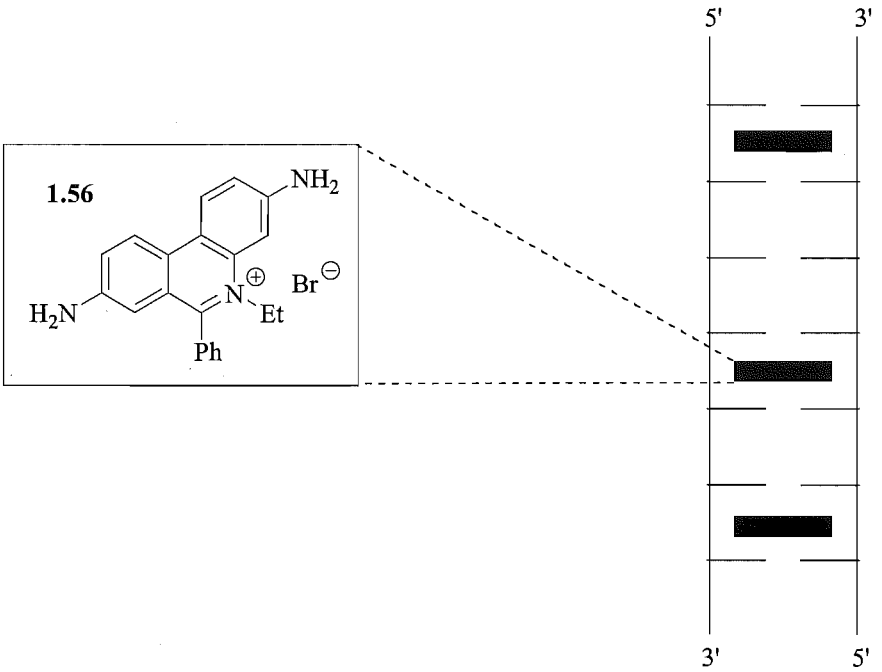


Figure 1.16. Interaction of ethidium bromide (**1.56**) with DNA. The double helix is represented in an unwound state for clarity.

An enormous array of intercalating compounds with antitumour properties have been identified over the last few decades through isolation from natural sources or by synthetic means, with many reaching clinical trial.[74] The compounds displayed in Figure 1.17 are some representative examples of intercalators with interesting activities and/or structural motifs.

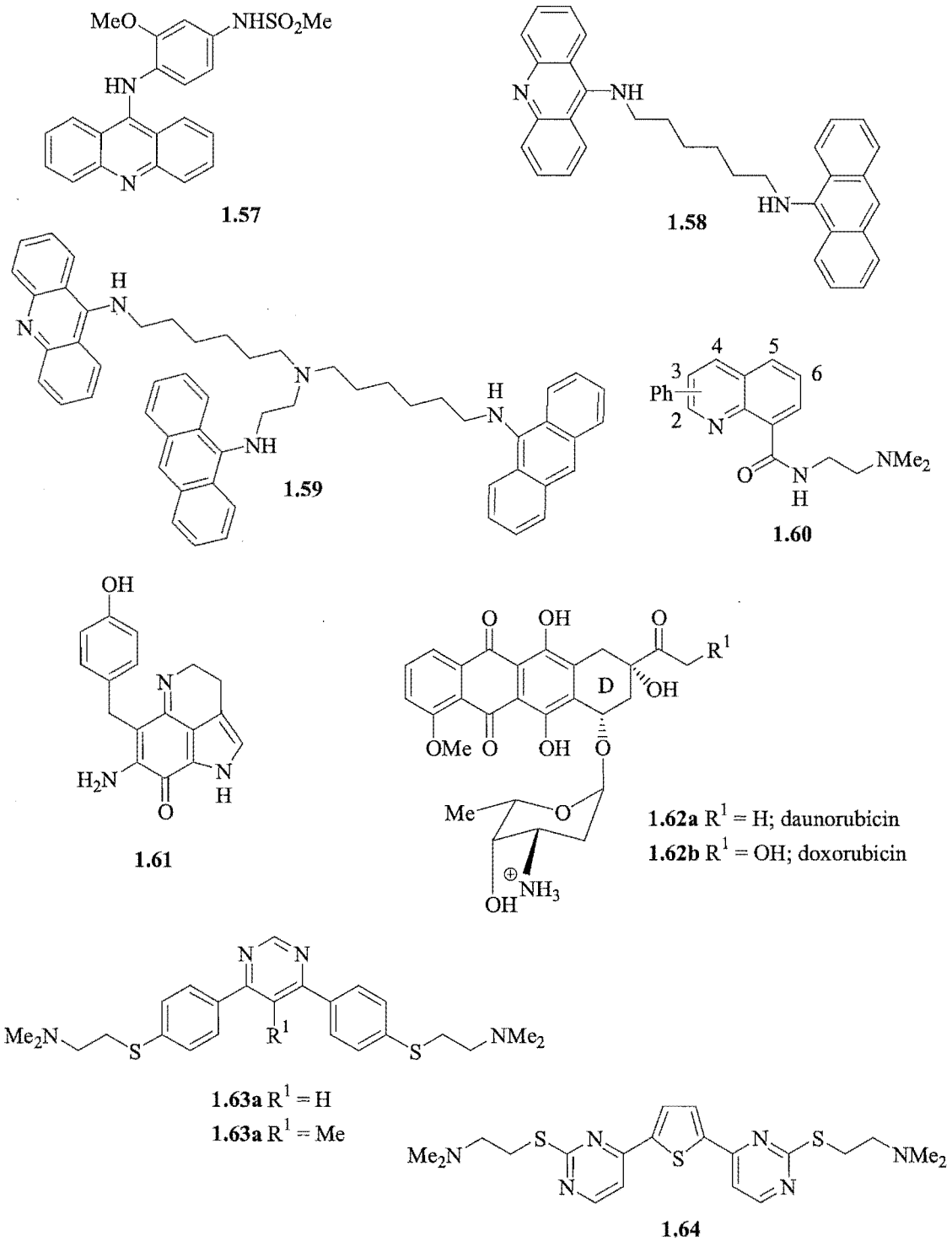


Figure 1.17. Structures of amsacrine (**1.57**), acridine-based bis- and tris-intercalators (**1.58-1.59**), a representative phenylquinoline-8-carboxamide (**1.60**), veiutamine (**1.61**), daunorubicin (**1.62a**), doxorubicin (**1.62b**), and unfused aromatic intercalators (**1.63a-b**, **1.64**).

Amsacrine (**1.57**) was the first synthetic DNA intercalating drug used clinically.[75] The bis- and tris-intercalators **1.58**[76] and **1.59**[77] were shown to insert into the double helix at more than one location per molecule. A series of phenylquinoline-8-carboxamides represented by **1.60** intercalated DNA if the phenyl ring was appended at the C2, C3 or C6 positions, but not if it was at positions C4 and C5.[78] Veiutamine (**1.61**) is an example of a natural product extracted from a marine source (the sponge *Zyzzya fuliginosa*), and it was found to be a potent *in vitro* cytotoxin.[79] The anthracycline class of antibiotics has yielded a range of potent compounds for the treatment of cancer, with daunorubicin (**1.62a**) and doxorubicin (**1.62b**) two classical examples.[74b] Studies to determine the drug-DNA interaction has revealed that the aglycone chromophore possessed by compounds of type **1.62** inserted into the double helix, while ring D and the amino sugar resided in the minor groove.[80] The unfused aromatics **1.63a-b**[81] and **1.64**[82] both interact with DNA in an intercalative fashion courtesy of the relatively planar structure of these agents. Compound **1.64** also amplified the bleomycin-mediated degradation of DNA.

The following section introduces the concept of "intercalating alkylators", *i.e.* compounds that possess an alkylating moiety fused to an intercalator to effect potent antitumour activity.

1.3.1 Intercalators that alkylate DNA via a reactive functionality

Many clinically useful alkylating agents display a moderate affinity towards tumour cells, however further improvements in specificity are considered desirable.[74b] An increased affinity for genetic material been effected by attachment of an intercalating ligand to an electrophile, the activity of which is reliant on its latent reactivity (*cf* **1.51**). The intercalators preference for inserting between the base pairs of the double helix presents the electrophilic moiety to a reactive site in the DNA. An increase in selectivity for the genetic material would help to overcome the problems of excessive reactivity associated with many DNA alkylators, as a high proportion of the anticancer agent can degrade *in vivo* by hydrolysis or reaction with other biomolecules *e.g.* proteins.[74b]

The nitrogen mustard **1.65** (Figure 1.18), which displayed cytotoxicity towards the P388 murine leukaemia cell line,[83] illustrates the bifunctional nature of these "intercalating alkylators". An acridine ring system acted as the DNA-directing moiety to deliver the alkyl halide functionality (which was attached to the intercalator via an alkyl linker) into close proximity to the DNA. As a result, there is a greater likelihood that the alkyl chloride moieties would react with the genetic material than other biomolecules.

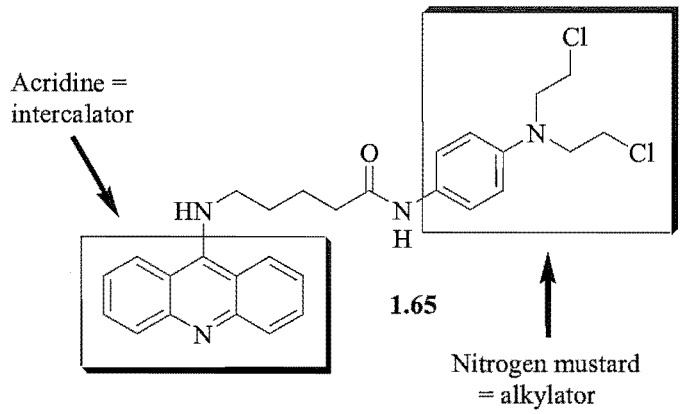


Figure 1.18. Structure of **1.65**, with the intercalating and alkylating functionalities highlighted.

An example of an intercalating alkylator with improved activity in comparison to its non-intercalating counterpart was reported by Jones, *et al.*[84] The pyrrolophenanthrene nucleus of **1.66** (Figure 1.19), which demonstrated intercalative activity, gave a 10-fold increase in potency against the HCT-116 colon cancer cell line over **1.67**, which only had a phenyl ring attached to the nitrogen mustard.

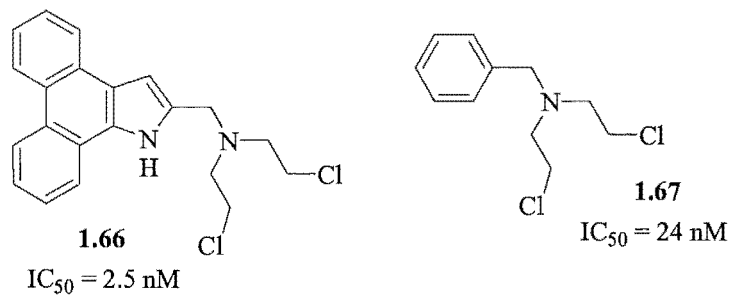


Figure 1.19. Structure and anticancer cell line activity of the bifunctional alkylating agents **1.66** (intercalating) and **1.67** (non-intercalating).

Denny *et al* described a comparison between the effectiveness of an intercalating (acridine) or minor groove binding (quinoline) carrier to effect cytotoxicity in the P388 cell line.[85] A range of compounds was synthesised, whereby the carrier was appended to an imidazole-, thioimidazole- or pyrrolizine-based alkylating unit. Subsequent analysis determined that the pyrrolizine-based unit was the most potent alkylator. The hydroxymethyl functionality highlighted in **1.68** (Figure 1.20) was resolved to be the point of attachment for pyrrolizines such as **1.68** to DNA. Analysis of the IC_{50} data against the P388 cell line demonstrated that greater activity was elicited by the minor groove binder **1.68** (IC_{50} = 0.03 μM) than the intercalator **1.69** (IC_{50} = 1.3 μM). Both compounds showed greater activity than the standard alkylating unit **1.70** (IC_{50} = >20 μM).

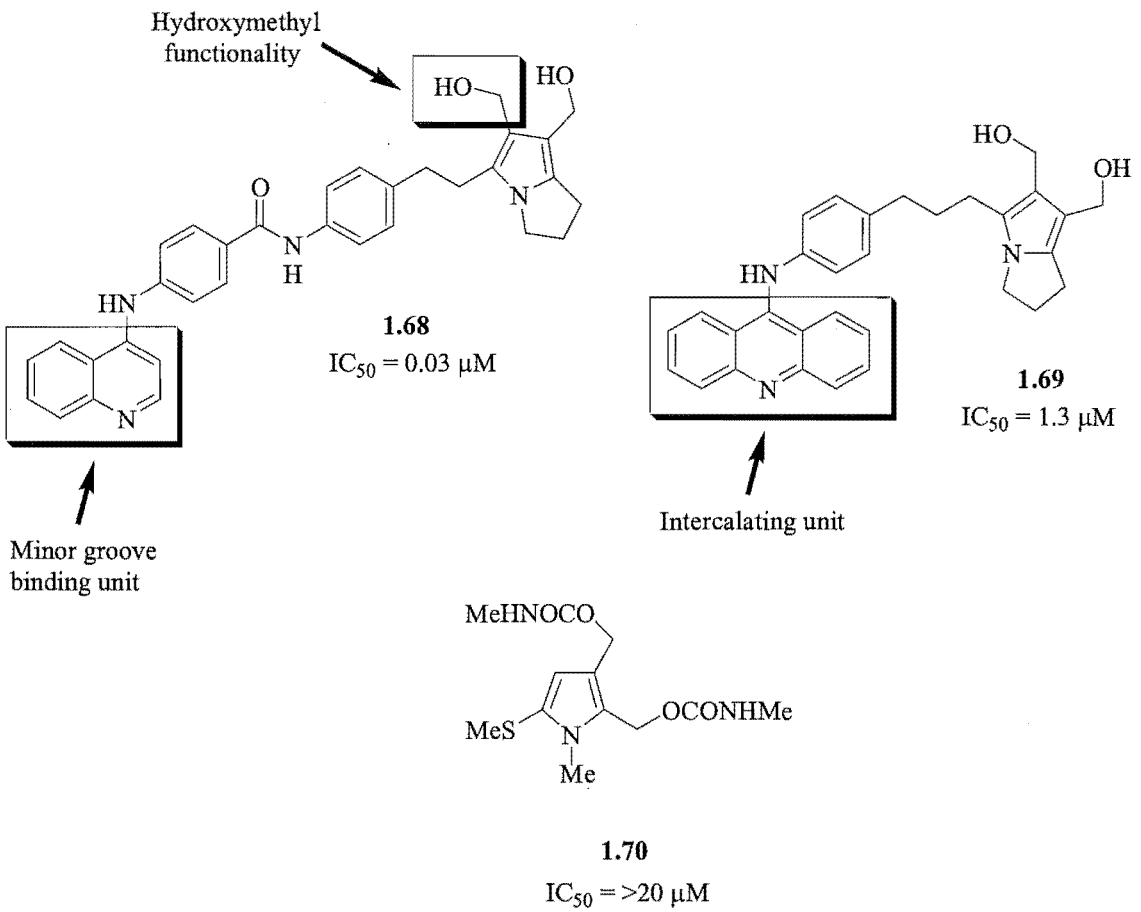
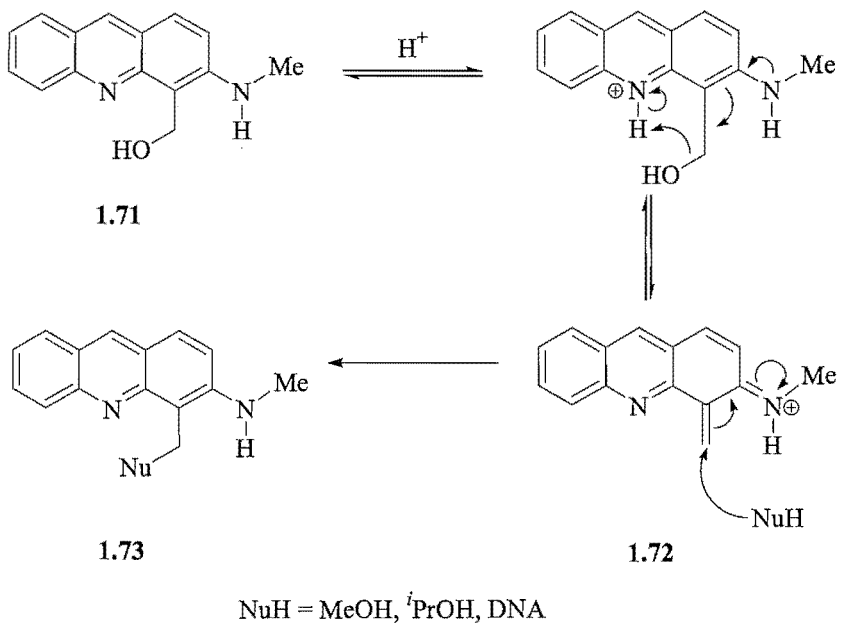


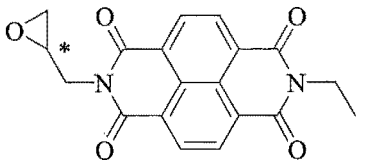
Figure 1.20. Structure and anticancer cell line activity of compounds **1.68** (minor groove binding alkylator), **1.69** (intercalating alkylator) and **1.70** (alkylator).

Another example of DNA alkylation mediated by a reactive hydroxymethyl group was reported by Demeunynck *et al.*[86] A range of compounds was prepared with the hydroxymethyl group directly attached to the intercalator. This is in contrast to **1.69**, where both an alkyl linker and a heterocycle separate the reactive functionality and intercalative moiety. Initial UV-visible spectroscopic analysis determined that a covalent bond was formed between **1.71** (Scheme 1.12) and calf-thymus DNA. Subsequent studies to elucidate the electrophilic nature of this class of compounds illustrated that the reaction proceeds by the mechanism illustrated in Scheme 1.12. The latent reactivity possessed by the protonated form of **1.71** was released upon formation of the quinone-imine-methide **1.72**, and reaction of **1.72** with a suitable nucleophile gave the adduct **1.73**.



Scheme 1.12. Reaction of **1.71** with various nucleophiles to form adduct **1.73**.

Selectivity for a specific nucleotide sequence was observed for the epoxide **1.74**[87] (Figure 1.21). The (*R*)-epoxide alkylated at the 3' guanine of a GG sequence, whereas the (*S*)-epoxide alkylated at the 5' guanine of that particular sequence. DNA affinity was conferred by the naphthyldiimide intercalator. This result illustrates that a degree of sequence selectivity can be incorporated into the structure of an intercalating alkylator.

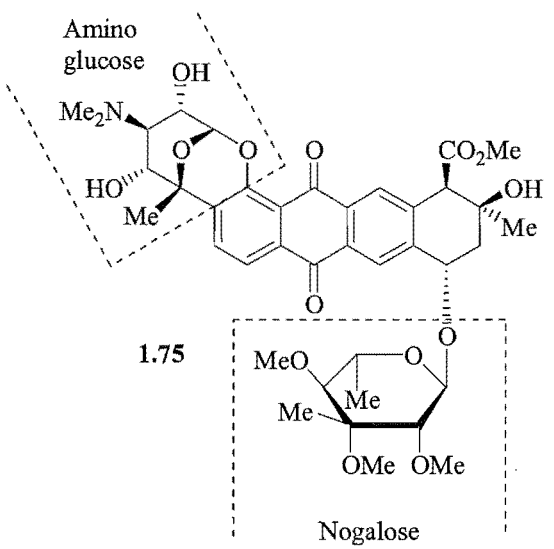


1.74

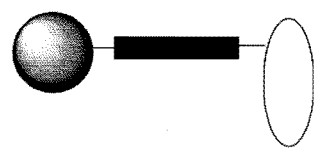
Figure 1.21. Structure of the intercalating alkylator **1.74**. The stereocentre is marked *.

1.3.2 Threading intercalators

One class of DNA intercalators is distinguished by the presence of non-intercalative functionalities at the distal ends of a planar aromatic structure. A novel mode of binding, known as "threading intercalation", occurs when the intercalator simultaneously directs the two substituents separately into each groove of the double helix.[88] The representative example nogalamycin (**1.75**)[89] is illustrated in Figure 1.22, in addition to its schematic representation **1.76**. Note the presence of the amino glucose (grey circle in **1.76**) and nogalose (white oval in **1.76**) sugar moieties attached to the intercalator.



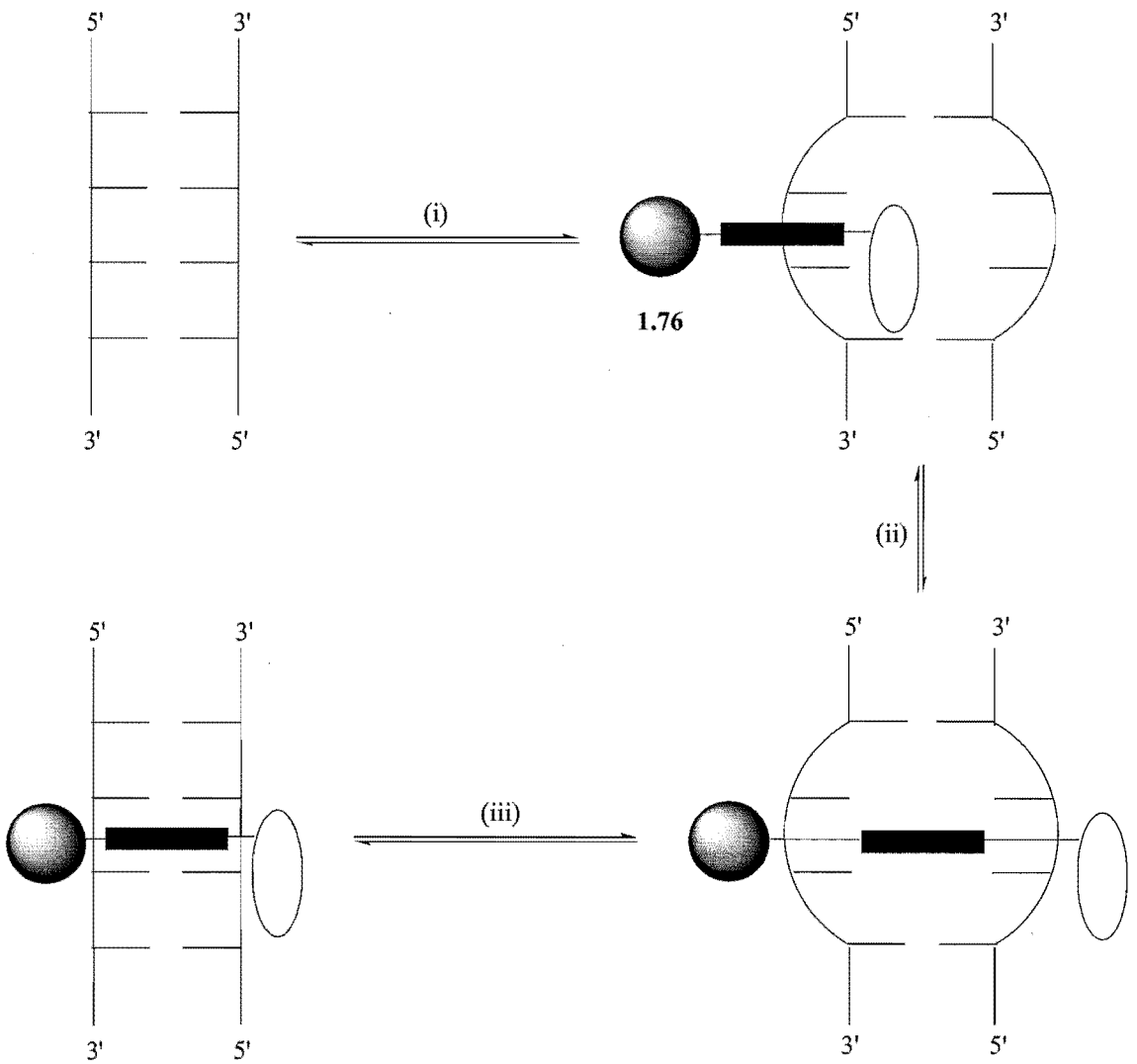
1.75



1.76

Figure 1.22. Structure of nogalamycin (**1.75**) and its schematic representation **1.76**.

Scheme 1.13 illustrates how threading intercalators such as nogalamycin interact with DNA. In step (i), the helix forms a temporary cavity by breaking hydrogen bonds between pairs of complementary bases. This enables the first non-intercalative moiety of **1.76** to "thread" through the base pairs and then occupy its preferred position in either the major or minor groove. The intercalator then sits between the base pairs of the distorted helix as a result of step (ii). In step (iii), the DNA reforms the hydrogen bonds that were disrupted temporarily to give a stable complex. Because the threading process in step (i) is relatively



Scheme 1.13. Schematic model for the threading intercalation of nogalamycin (represented by **1.76**) to DNA.

disfavoured, the binding constants for compounds of this type are expected to be lower than that of mono-substituted intercalators. Conversely, the dissociation rate (essentially step (i) in reverse) is also expected to be lower, so a greater retention time within DNA is expected for these compounds.[72a]

Further examples of threading intercalators are displayed in Figure 1.23. The naphthalenediimide **1.77**, with distal alkylamino substituents, bound polyGC sequences 20-25 times more strongly than polyAT sequences.[72a] This result demonstrated that sequence specificity can be incorporated into threading intercalators. Compound **1.78** was synthesised by solid phase synthesis and utilised amino acid side chains to interact with the grooves of the double helix.[88] A threading intercalator was created by appending cyclopropapyrroloindole (CPI) and triplex-forming oligonucleotide (TFO) moieties to an acridine nucleus to afford **1.79**.[90] The CPI unit binds to the minor groove of DNA, while the TFO resides in the major groove. A novel threading intercalator binding mode was exhibited by **1.80**, and it bound to DNA in the manner represented by **1.81**.[91]

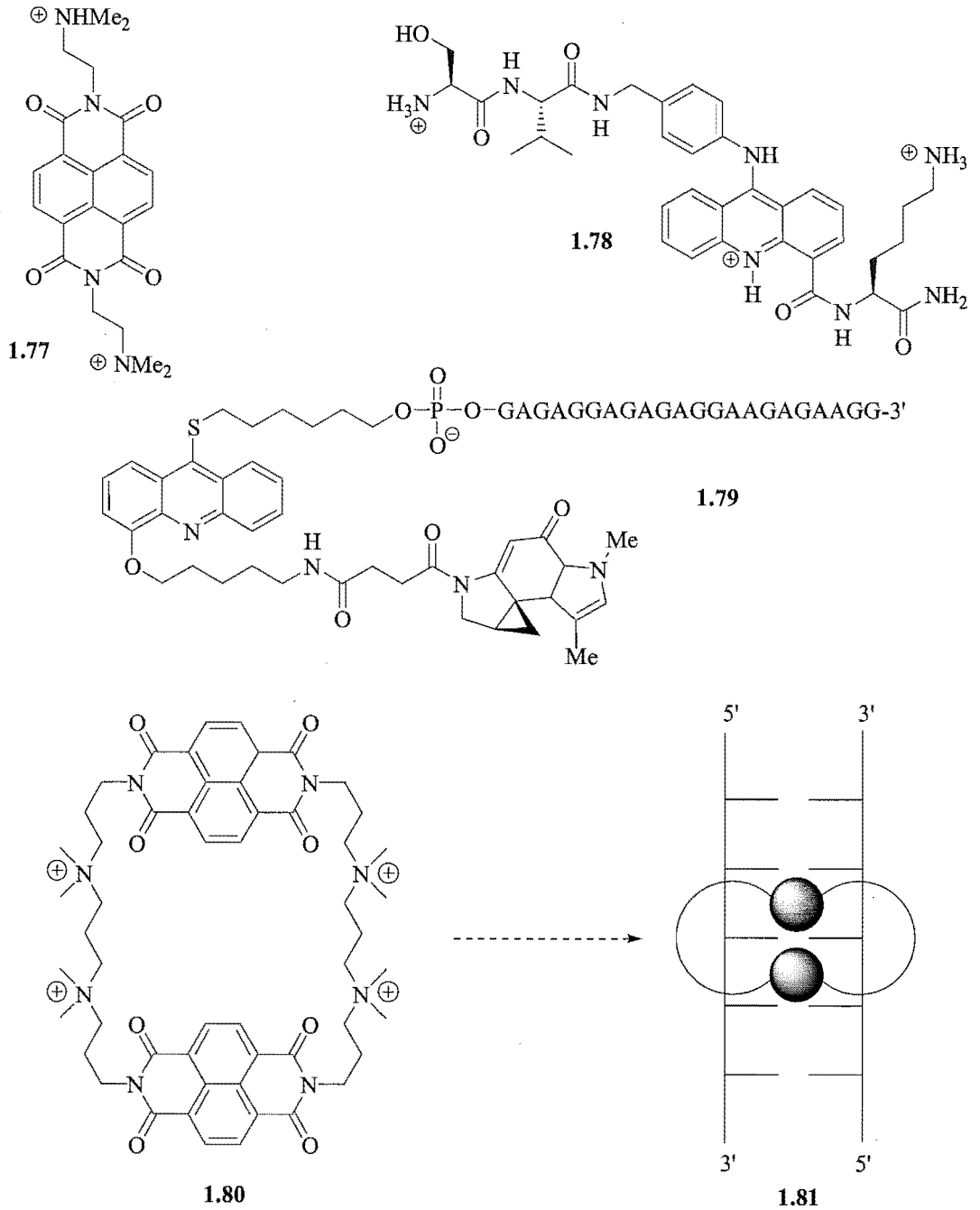


Figure 1.23. Representative threading intercalators **1.77-1.80**.

1.4 WORK DESCRIBED IN THIS THESIS

The modulation of a compounds chemical or physical properties at the site of action can give rise to highly specific and potent interactions with a biological target. This thesis addresses the application of nitrogen-based heterocycles that possess latent reactivity towards two biological targets - the inhibition of α-chymotrypsin, and the development of putative anticancer compounds.

Chapter Two describes the attempts made to elicit metal-enhanced enzyme inhibition against α-chymotrypsin. This enzyme is considered an excellent model system for serine protease inhibition due to its well defined active site structure and substrate specificity, in addition to being an important step in the development of inhibitors for chymotrypsin-like enzymes *e.g.* cathepsin G, mast cell chymase and human leukocyte elastase. The inhibitory activity of these compounds was proposed to be enhanced by the formation of a ternary complex between the enzyme, inhibitor and a metal ion. Incorporation of an aromatic functionality into the structure of the prepared inhibitors was considered critical for eliciting inhibition. The inhibition of α-chymotrypsin with or without the presence of Zn^{2+} was determined to elucidate whether the synthesised analogues inhibited the target enzyme in a metal-mediated fashion.

Chapter Three describes the synthesis and assay of C5-(hydroxyalkyl)pyrroles as potential mechanism-based α-chymotrypsin inhibitors. Reaction of the C5-(hydroxyalkyl)pyrrole in the enzyme active site was proposed to unmask a reactive azafulvene species, which would subsequently form a covalent bond to the enzyme. Deactivation by a less electron-withdrawing C2-acyl group was projected to increase the probability of azafulvene formation, relative to previously prepared inhibitors with an electron-withdrawing group on the pyrrole ring nitrogen, and hence result in a more reactive series of compounds. Emphasis was placed on the optimisation of inhibition by modification at positions C2 and C5. The application of solid phase organic synthesis toward the preparation of this class of compounds was also attempted.

Chapter Four describes the derivatisation of a series of pyrrole-amino acid adducts prepared in Chapter Three under mild conditions to give a range of pyrrolizinones. This sequence of reactions was proposed to be a novel two-step method for initially derivatising the amino group of an amino acid, after which latent spectrophotometric properties were released upon pyrrolizinone formation. Investigations were performed to determine whether the nature of the amino acid side chain adjacent to the pyrrolizinone ring system could be elucidated by UV spectroscopy or low resolution mass spectrometry.

Chapter Five describes the attempted development of a novel series of compounds with potential antitumour activity. The proposed mechanism of action involves reaction of a 5-(hydroxymethyl)pyrrole functionality with the nucleophilic sites present in DNA, in a manner analogous that that of the C5-(hydroxyalkyl)pyrrole α-chymotrypsin inhibitors described in Chapter Two. A proposed increase in affinity toward DNA was to be endowed upon the 5-(hydroxymethyl)pyrrole alkylators by the presence of an attached intercalative moiety.

1.5 REFERENCES FOR CHAPTER ONE

1. Mann, J. In *Secondary Metabolism*; Oxford University Press: Oxford, 1978, pp. 79-151.

2. Henschke, J. P.; Rickards, R. W. *Tetrahedron Lett.* **1996**, *37*, 3557.

3. Friedlos, F.; Denny, W. A.; Palmer, B. D.; Springer, C. J. *J. Med. Chem.* **1997**, *40*, 1270 and references therein.

4. Harvey, A. J.; Abell, A. D. *Tetrahedron* **2000**, *56*, 9763.

5 Walsh, C. In *Enzymatic Reaction Mechanisms*; W. H. Freeman and Company: San Francisco, 1979, pp. 53-107.

6. Kenny, A. J. In *Proteolytic Enzymes: Tools and Targets*; Sterchi, E. E., Stocker, W., Eds.; Springer-Verlag: Berlin, 1999, pp. 1-8.

7. (a) Hedstrom, L. *Chem. Rev.* **2002**, *102*, 4501. (b) Neurath, H. *Science* **1984**, *224*, 350.

8. Stroud, R. M.; Krieger, M.; Koeppe III, R. E.; Kossiakoff, A. A.; Chambers, J. L. *Cold Spring Harbor Conferences on Cell Proliferation* **1975**, *2*, 13.

9. Stroud, R. M. *Sci. Amer.* **1974**, *231*, 74.

10. Perona, J. J.; Craik, C. S. *Prot. Sci.* **1995**, *4*, 337.

11. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.

12. Laskowski, J., M.; Tashiro, M.; Empie, M. W.; Park, S. J.; Kato, I.; Ardelt, W.; Wieczorek, M. In *Proteinase Inhibitors: Medical and Biological Aspects*; Katunuma, N., Umezawa, H., Holzer, H., Eds.; Springer-Verlag: Tokyo, 1983, pp. 55-68.

13. (a) Horl, W. H.; Heidland, A. E. *Proteases II: Potential Role in Health and Disease*; Plenum Press: New York, 1988; Vol. 240. (b) Groutas, W. C. *Med. Res. Rev.* **1987**, *7*, 227. (c) Groutas, W. C.; Giri, P. K.; Crowley, J. P.; Castrisos, J. C.; Brubaker, M. J. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 741. (d) Pochet, L.; Doucet, C.; Dive, G.; Wouters, J.; Masareel, B.; Reboud-Ravaux, M.; Pirotte, B. *Bioorg. Med. Chem.* **2000**, *8*, 1489.

14. Katz, B. A.; Stroud, R. M.; Clark, J. M.; Jenkins, T. E.; Janc, J. W.; Moore, W. R.; Venuti, M. C. *Special Publication - Royal Society of Chemistry* **2001**, *264 (Medicinal Chemistry into the New Millenium)*, 211.

15. Walsh, C. *Tetrahedron* **1982**, *38*, 871.

16. (a) Abell, A. D. *Lett. Pep. Sci.* **2002**, *8*, 267. (b) Abell, A. D.; Gardiner, J. *J. Org. Chem.* **1999**, *64*, 9668. (c) Thompson, S. K.; Eppley, A. M.; Frazee, J. S.; Darcy, M. G.; Lum, R. T.; Tomaszek, J. T. A.; Ivanoff, L. A.; Morris, J. F.; Sternberg, E. J.; Lambert, D. M.; Fernandez, A. V.; Petteway, J. S. R.; Meek, T. D.; Metcalf, B. W.; Gleason, J. G. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2441. (d) Dreyer, G. B.; Gleason, J. G.; Meek, T. D.; Thompson, S. K.; SmithKline Beecham Corp., USA: PCT Int. Appl., 1993.

17. (a) Henderson, R.; Wright, C. S.; Hess, G. P.; Blow, D. M. *Cold Spring Harbor Symposia on Quantitative Biology* **1971**, *36*, 63. (b) Sigler, P. B.; Blow, D. M.; Matthews, B. W.; Henderson, R. *J. Mol. Biol.* **1968**, *35*, 143. (c) Matthews, B. W.; Sigler, P. B.; Henderson, R.; Blow, D. M. *Nature* **1967**, *214*, 652.

18. Kashima, A.; Inoue, Y.; Sugio, S.; Maeda, I.; Nose, T.; Shimohigashi, Y. *Eur. J. Biochem.* **1998**, *255*, 12.

19. Katz, B. A.; Clark, J. M.; Finer-Moore, J. S.; Jenkins, T. E.; Johnson, C. R.; Ross, M. J.; Luong, C.; Moore, W. R.; Stroud, R. M. *Nature* **1998**, *391*, 608.

20. Knight, C. G. In *Proteolytic Enzymes: Tools and Targets*; Sterchi, E. E., Stocker, W., Eds.; Springer-Verlag: Berlin, 1999, pp. 167-187.

21. Janc, J. W.; Clark, J. M.; Warne, R. L.; Elrod, K. C.; Katz, B. A.; Moore, W. R. *Biochemistry* **2000**, *39*, 4792.

22. Yeung, K.-S.; Meanwell, N. A.; Qiu, Z.; Hernandez, D.; Zhang, S.; McPhee, F.; Weinheimer, S.; Clark, J. M.; Janc, J. W. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2355.

23. Sperandio, D.; Gangloff, A. R.; Litvak, J.; Goldsmith, R.; Hataye, J. M.; Wang, V. R.; Shelton, E. J.; Elrod, K.; Janc, J. W.; Clark, J. M.; Rice, K.; Weinheimer, S.; Yeung, K.-S.; Meanwell, N. A.; Hernandez, D.; Staab, A. J.; Venables, B. L.; Spencer, J. R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3129.

24. Bartenschlager, R.; Lohmann, V. *J. Gen. Virol.* **2000**, *81*, 1631.

25. Keleti, T. In *Basic Enzyme Kinetics*; Akademiai Kiado: Budapest, 1986, pp. 125-402.

26. Dhanak, D.; Burton, G.; Christmann, L. T.; Darcy, M. G.; Elrod, K. C.; Kaura, A.; Keenan, R. M.; Link, J. O.; Peishoff, C. E.; Shah, D. H. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2279.

27. Toyota, E.; Ng, K. K. S.; Sekizaki, H.; Itoh, K.; Tanizawa, K.; James, M. N. G. *J. Mol. Biol.* **2001**, *305*, 471.

28. Brinkerhoff, C. J.; Podsiadlo, P.; Komiyama, T.; Fuller, R. S.; Blum, O. *ChemBioChem* **2002**, *3*, 1141.

29. Rockwell, N. C.; Krysan, D. J.; Komiyama, T.; Fuller, R. S. *Chem. Rev.* **2002**, *102*, 4525.

30. Molloy, S. S.; Anderson, E. D.; Jean, F.; Thomas, G. *Trends Cell Biol.* **1999**, *9*, 28.

31. Presnell, S. R.; Patil, G. S.; Mura, C.; Jude, K. M.; Conley, J. M.; Bertrand, J. A.; Kam, C.-M.; Powers, J. C.; Williams, L. D. *Biochemistry* **1998**, *37*, 17068.

32. Liang, X.; Parkinson, J. A.; Weishaeupl, M.; Gould, R. O.; Paisey, S. J.; Park, H.-S.; Hunter, T. M.; Blindauer, C. A.; Parsons, S.; Sadler, P. J. *J. Am. Chem. Soc.* **2002**, *124*, 9105.

33. Basmaciogullari, S.; Babcock, G. J.; Van Ryk, D.; Wojtowicz, W.; Sodroski, J. *J. Virol.* **2002**, *76*, 10791.

34. Gerlach, L. O.; Jakobsen, J. S.; Jensen, K. P.; Rosenkilde, M. R.; Skerlj, R. T.; Ryde, U.; Bridger, G. J.; Schwartz, T. W. *Biochemistry* **2003**, *42*, 710.

35. Valente, S.; Gobbo, M.; Licini, G.; Scarso, A.; Scrimin, P. *Angew. Chem. Int. Ed.* **2001**, *40*, 3899.

36. DeCamp, D. L.; Babe, L. M.; Salto, R.; Lucich, J. L.; Koo, M.-S.; Kahl, S. B.; Craik, C. S. *J. Med. Chem.* **1992**, *35*, 3426.

37. Davis, D. A.; Branca, A. A.; Pallenberg, A. J.; Marschner, T. M.; Patt, L. M.; Chatlynne, L. G.; Humphrey, R. W.; Yarchoan, R.; Levine, R. L. *Arch. Biochem. Biophys.* **1995**, *322*, 127.

38. Lebon, F.; De Rosny, E.; Reboud-Ravaux, M.; Durant, F. *Eur. J. Med. Chem.* **1998**, *33*, 733.

39. Lebon, F.; Boggetto, N.; Ledecq, M.; Durant, F.; Benatallah, Z.; Sicsic, S.; Lapouyade, R.; Kahn, O.; Mouithys-Mickalad, A.; Deby-Dupont, G.; Reboud-Ravaux, M. *Biochem. Pharm.* **2002**, *63*, 1863.

40. Hanson, M. A.; Oost, T. K.; Sukonpan, C.; Rich, D. H.; Stevens, R. C. *J. Am. Chem. Soc.* **2000**, *122*, 11268.

41. Sathyamoorthy, V.; Dasgupta, B. R. *J. Biol. Chem.* **1985**, *260*, 10461.

42. Eswaramoorthy, S.; Kumaran, D.; Swaminathan, S. *Biochemistry* **2002**, *41*, 9795.

43. Nguyen, R.; Huc, I. *Angew. Chem. Int. Ed.* **2001**, *40*, 1774.

44. ul-Hassan, M.; Scozzafava, A.; Chohan, Z. H.; Supuran, C. T. *J. Enz. Inhib.* **2001**, *16*, 499.

45. Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2667.

46. (a) Levy, D. E.; Marlowe, C.; Kane-Maguire, K.; Bao, M.; Cherbavaz, D. B.; Tomlinson, J. E.; Sedlock, D. M.; Scarborough, R. M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3085. (b) Levy, D. E.; Bao, M.; Tomlinson, J. E.; Scarborough, R. M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3089.

47. Krupinski, J. *Mol. Cell. Biochem.* **1991**, *104*, 73.

48. Wu, P.; Leinonen, J.; Koivunen, E.; Lankinen, H.; Stenman, U.-H. *Eur. J. Biochem.* **2000**, *267*, 6212.

49. Elling, C. E.; Thirstrup, K.; Holst, B.; Schwartz, T. W. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12322.

50. Abeles, R. H. In *Enzyme-Activated Irreversible Inhibitors*; Seiler, N., Jung, M. J., Koch-Weser, J., Eds.; Elsevier North-Holland Inc: Amsterdam, 1978, pp. 1-12.

51. Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press, Inc.: Boca Raton, 1988; Vol. I and II.

52. Seiler, N.; Jung, M. J.; Koch-Weser, J. *Enzyme-Activated Irreversible Inhibitors*; Elsevier North-Holland Inc.: Amsterdam, 1978.

53. Abell, A. D. In *Advances in Detailed Reaction Mechanisms*; Coxon, J. M., Ed.; JAI Press Inc.: Greenwich, 1992; Vol. 2, pp. 243-277.

54. Harper, J. W.; Hemmi, K.; Powers, J. C. *Biochemistry* **1985**, *24*, 1831.

55. Gelb, M. H.; Abeles, R. H. *Biochemistry* **1984**, *23*, 6596.

56. Hedstrom, L.; Moorman, A. R.; Dobbs, J.; Abeles, R. H. *Biochemistry* **1984**, *23*, 1753.

57. Moorman, A. R.; Abeles, R. H. *J. Am. Chem. Soc.* **1982**, *104*, 6785.

58. Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1983**, *258*, 15046.

59. Rai, R.; Katzenellenbogen, J. A. *J. Med. Chem.* **1992**, *35*, 4150.

60. Altman, R.; Sarg, M. J. *The Cancer Dictionary*; Facts On File, Inc.: New York, 1992.

61. Kornberg, A.; Baker, T. A. *DNA Replication*; 2nd ed.; W. H. Freeman and Company: New York, 1992.

62. Goodsell, D. S. *Oncologist* **2002**, *7*, 381.

63. Wang, J. C. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 430.

64. Bakshi, R. P.; Galande, S.; Muniyappa, K. *Crit. Rev. Biochem. Mol. Biol.* **2001**, *36*, 1.

65. Froelich-Ammon, S. J.; Osheroff, N. *J. Biol. Chem.* **1995**, *270*, 21429.

66. Heck, M. M. S.; Earnshaw, W. C. *J. Cell Biol.* **1986**, *103*, 2569.

67. Hartley, J. A. In *Molecular Aspects of Anticancer Drug-DNA Interactions*; Neidle, S., Waring, M., Eds.; CRC Press Inc.: Boca Raton, 1993; Vol. 1, pp. 1-31.

68. Lawley, P. D.; Phillips, D. H. *Mutat. Res.* **1996**, *355*, 13.

69. Everett, J. L.; Roberts, J. J.; Ross, W. C. J. *J. Chem. Soc.* **1953**, 2386.

70. Lerman, L. S. *J. Mol. Biol.* **1961**, *3*, 18.

71. LePecq, J.-B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87.

72. (a) Tanious, F. A.; Yen, S.-F.; Wilson, W. D. *Biochemistry* **1991**, *30*, 1813. (b) Collier, D. A.; Neidle, S.; Brown, J. R. *Biochem. Pharm.* **1984**, *33*, 2877.

73. (a) Robinson, M. J.; Osheroff, N. *Biochemistry* **1991**, *30*, 1807. (b) De Isabella, P.; Capranico, G.; Zunino, F. *Life Sci.* **1991**, *48*, 2195.

74. (a) Denny, W. A. In *Cancer Chemotherapeutic Agents*; Foye, W. O., Ed.; American Chemical Society: Washington, D. C., 1995, pp. 218-239. (b) Denny, W. A. *Anticancer Drug Des.* **1989**, *4*, 241. (c) Rewcastle, G. W.; Denny, W. A. *Chem. N.Z.* **2002**, *66*, 22. (d) Ding, Q.; Chichak, K.; Lown, J. W. *Curr. Med. Chem.* **1999**, *6*, 1. (e) Wang, A. H.-J. In *Molecular Aspects of Anticancer Drug-DNA Interactions*; Neidle, S., Waring, M., Eds.; CRC Press Inc.: Boca Raton, 1993; Vol. 1, pp. 32-53. (f) Romerdahl, C. A.; Brana, M. F. In *Cancer Therapeutics: Experimental and Clinical Agents*; Teicher, B. A., Ed.; Humana Press Inc.: Totowa, N.J., 1997, pp. 215-226. (g) Vogel, P. *Curr. Org. Chem.* **1998**, *2*, 255.

75. Cain, B. F.; Atwell, G. J. *Eur. J. Cancer* **1974**, *10*, 539.

76. Denny, W. A.; Atwell, G. J.; Baguley, B. C.; Wakelin, L. P. G. *J. Med. Chem.* **1985**, *28*, 1568.

77. Hansen, J. B.; Koch, T.; Buchardt, O.; Nielsen, P. E.; Norden, B.; Wirth, M. *J. Chem. Soc., Chem. Commun.* **1984**, 509.

78. Atwell, G. J.; Bos, C. D.; Baguley, B. C.; Denny, W. A. *J. Med. Chem.* **1988**, *31*, 1048.

79. Venables, D. A.; Barrows, L. R.; Lassota, P.; Ireland, C. M. *Tetrahedron Lett.* **1997**, *38*, 721.

80. Wang, A. H. J.; Ughetto, G.; Quigley, G. J.; Rich, A. *Biochemistry* **1987**, *26*, 1152.

81. Wilson, W. D.; Strekowski, L.; Tanious, F. A.; Watson, R. A.; Mokrosz, J. L.; Strekowska, A.; Webster, G. D.; Neidle, S. *J. Am. Chem. Soc.* **1988**, *110*, 8292.

82. Strekowski, L.; Strekowska, A.; Watson, R. A.; Tanious, F. A.; Nguyen, L. T.; Wilson, W. D. *J. Med. Chem.* **1987**, *30*, 1415.

83. Gourdie, T. A.; Valu, K. K.; Gravatt, G. L.; Boritzki, T. J.; Baguley, B. C.; Wakelin, L. P. G.; Wilson, W. R.; Woodgate, P. D.; Denny, W. A. *J. Med. Chem.* **1990**, *33*, 1177.

84. Jones, G. B.; Mathews, J. E. *Tetrahedron* **1997**, *53*, 14599.

85. Atwell, G. J.; Fan, J.-Y.; Tan, K.; Denny, W. A. *J. Med. Chem.* **1998**, *41*, 4744.

86. Charmantray, F.; Duflos, A.; Lhomme, J.; Demeunynck, M. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2962.

87. Okamoto, A.; Nakamura, T.; Yoshida, K.; Nakatani, K.; Saito, I. *Org. Lett.* **2000**, *2*, 3249.

88. Carlson, C. B.; Beal, P. A. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1979.

89. Takenaka, S.; Takagi, M. *Bull. Chem. Soc. Jpn.* **1999**, *72*, 327.

90. Dempcy, R. O.; Kutyavin, I. V.; Mills, A. G.; Lukhtanov, E. A.; Meyer, R. B. *Nucleic Acids Res.* **1999**, *27*, 2931.

91. Takagi, M.; Yokoyama, H.; Takenaka, S.; Yokoyama, M.; Kondo, H. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1998**, *32*, 375.

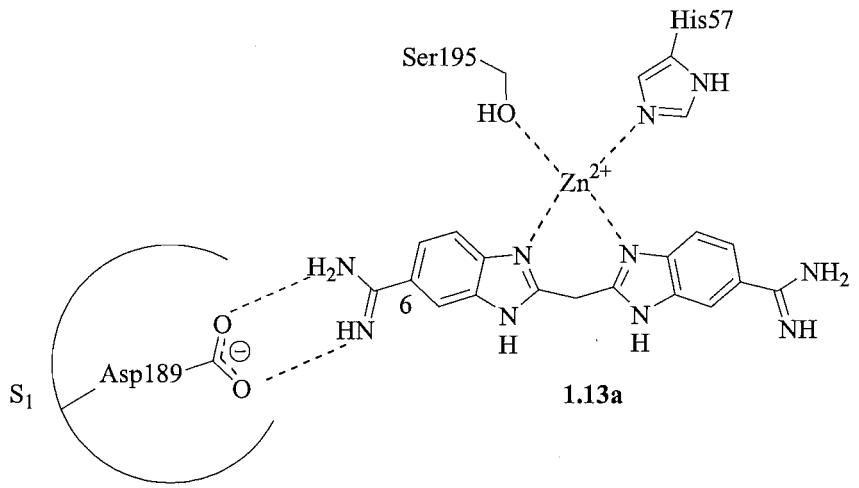
CHAPTER TWO

DEVELOPMENT OF ZINC-MEDIATED BENZIMIDAZOLE-BASED INHIBITORS OF α-CHYMOTRYPSIN

2.1 INTRODUCTION

There is an ongoing interest in these laboratories toward the development of heterocyclic-based inhibitors of α-chymotrypsin. This enzyme is considered an excellent model system for developing novel classes of serine protease inhibitors due to its well-characterised active site structure[1] and substrate specificity.[2] Additionally, inhibition of α-chymotrypsin is regarded as an important step toward the inhibition of enzymes with similar substrate specificity, such as cathepsin G, mast cell chymase and human leukocyte elastase.[3] These enzymes have been implicated in a number of debilitating diseases in the human body[4] (see Chapter One, Section 1.2 for further discussion of the disease states arising from serine protease over-activity).

A novel mode of serine protease inhibition, reported by Katz *et al*,[5] was of interest in these laboratories as a new approach towards the inhibition of α-chymotrypsin. BABIM (**1.13a**, Figure 2.1), a bis(benzimidazole)methane-based compound, was a moderate inhibitor of



1.13a

	K_i (EDTA), μM	K_i (Zn^{2+}), μM	Zn^{2+} factor
Trypsin	18.8	0.09	210
Chymotrypsin	710	120	6

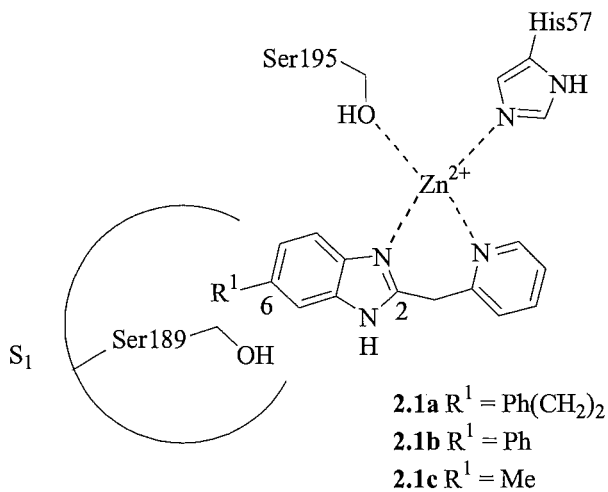
Figure 2.1. Ternary complex formed between **1.13a**, Zn^{2+} and trypsin, as determined by X-ray crystallography. The interaction between **1.13a** and the S_1 subsite of trypsin is illustrated. Inhibition data for **1.13a** against trypsin and chymotrypsin is also displayed.

trypsin (K_i = 18.8 mM) in the presence of EDTA, a metal ion complexing agent. However, upon the addition of Zn^{2+} the activity of **1.13a** against trypsin was increased by a "Zn^{2+} factor" of 210 to K_i = 90 nM. The ternary complex formed between **1.13a**, Zn^{2+} and trypsin, displayed in Figure 2.1, was determined by X-ray crystallography, and it provided a basis for these observations. Binding of **1.13a** to the enzyme active site residues Ser195 and His57, which are essential to the normal catalytic activity of the enzyme, was mediated by the metal ion. Therefore, complexation of Zn^{2+} by **1.13a** resulted in a more optimal fit in the active site of trypsin than that of **1.13a** alone, *i.e.* activity of the inhibitor is modulated by the presence of Zn^{2+}. This mode of binding is an important application of the concepts discussed in Chapter One, *i.e.* modulating the chemical or physical properties of a molecule to elicit a specific interaction with a biological target. A series of inhibitors analogous to **1.13a** were prepared and assayed for activity against a selection of serine proteases, and a wide range of inhibitor potencies and Zn^{2+} factors were observed (see Chapter One, Section 1.2.1 for further discussion). A common structural feature for compounds of type **1.13** was the presence of an amidine moiety at the C6 position. Formation of a salt bridge between the amidine group and Asp189 at the base of the S_1 subsite enhanced the activity displayed by compounds of type **1.13** towards trypsin-like enzymes. (See Chapter One, Section 1.2 for a discussion of the S/P nomenclature developed by Schechter and Berger.[6]) The amidine functionality mimics the basic arginine or lysine residues which occupy the P_1 position of trypsins natural substrate,[2b] which revealed that the C6 group was integral for recognition of the inhibitor by trypsin.

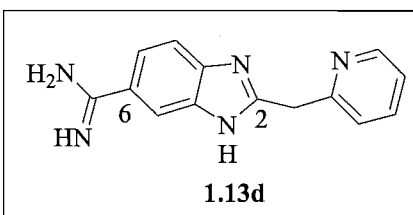
The mode of inhibition illustrated in Figure 2.1 stimulated research towards the inhibition of the target enzyme of these laboratories, α-chymotrypsin, in a manner analogous to that displayed by **1.13a** against trypsin. Relatively moderate inhibition, and a small enhancement of activity when Zn^{2+} was present, was displayed by **1.13a** against α-chymotrypsin (see data in Figure 2.1). The research discussed in this chapter focuses on two areas that would determine the extent to which this binding mechanism could be applied to other serine proteases. The first involved determination of whether a Zn^{2+}-mediated enhancement of inhibition, greater than that observed for **1.13a**, could be elicited against α-chymotrypsin. Secondly, the importance of the substituent at C6 would be

addressed. A hydrophobic substituent at C6 would endow greater affinity upon the putative inhibitor against α-chymotrypsin, in comparison to that observed for **1.13a**, as this enzyme has a predilection for substrates and inhibitors with aromatic substituents.[2a,7] This preference is a result of the Ser189 residue at the base of the non-polar S_1 subsite in chymotrypsin-like enzymes (*cf* Asp189 in trypsin).[5]

Three proposed α-chymotrypsin inhibitors (**2.1a-c**, Figure 2.2), based on a benzimidazole ring system, were initially targeted for synthesis. Figure 2.2 displays a possible ternary complex between inhibitors of type **2.1**, Zn^{2+} and the enzyme. The benzimidazole ring is substituted at C2 with a pyridin-2-ylmethyl moiety. As a result, compounds of type **2.1** can act as a bidentate ligand that occupies two of the four Zn^{2+} co-ordination sites. Residues Ser195 and His57 of the catalytic triad coordinate the two remaining sites. Occupation of the S_1 subsite with an aromatic moiety at the C6 position (*i.e.* R^1) of the benzimidazole nucleus was proposed to elicit a favourable interaction between inhibitors of type **2.1** and the enzyme, with a concomitant increase in activity. Compound **2.1a** has a 2-phenylethyl group at R^1, whereas **2.1b** possesses a phenyl group at the same position. The presence of the ethylene linker in R^1 of **2.1a** was expected to increase its flexibility, potentially resulting in a more optimal fit in the S_1 subsite than the phenyl group of **2.1b**.



2.1a $R^1 = Ph(CH_2)_2$
2.1b $R^1 = Ph$
2.1c $R^1 = Me$



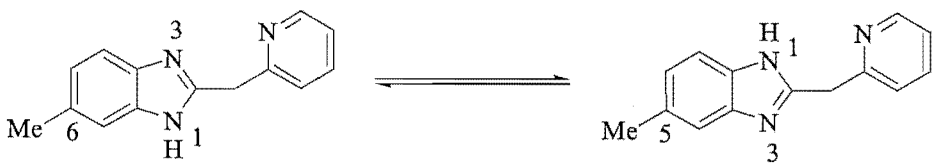
1.13d

Trypsin K_i (EDTA) = >1,000 μM
K_i (Zn^{2+}) = 136 μM
Zn^{2+} factor = >7

Figure 2.2. Structure of the proposed α-chymotrypsin inhibitors **2.1a-c**, and the anticipated mode of binding in the active site. Inhibitor **1.13d**, and its activity against trypsin, is highlighted for comparison.

Hence, **2.1a** may be expected to elicit greater activity against α-chymotrypsin than **2.1b**. Comparison of the activity displayed by benzimidazole **2.1c** (R^1 = Me) to that of **2.1a-b** would determine the importance of the aromatic R^1 groups possessed by **2.1a-b** for inhibitory activity. The ability of the 2-(pyridin-2-ylmethyl)benzimidazole nucleus to effect Zn^{2+}-enhanced inhibition was demonstrated by **1.13d** (highlighted in Figure 2.2), as it displayed a greater than 7-fold enhancement for trypsin inhibition when Zn^{2+} was present.[5]

The position of the aromatic functionality possessed by compounds of type **2.1** must be considered. For example, **2.1c** can exist as two tautomers, denoted as **2.1c(1)** or **2.1c(2)** in Figure 2.3, with the methyl group residing at either C6 or C5 respectively. Future representation of the proposed inhibitors will be that of **2.1c(1)**. This assumption was based on the tautomeric form of **1.13a,** which resided in the active site of trypsin with the amidine group at C6 as determined by X-ray crystallography[5] (see Figure 2.1).

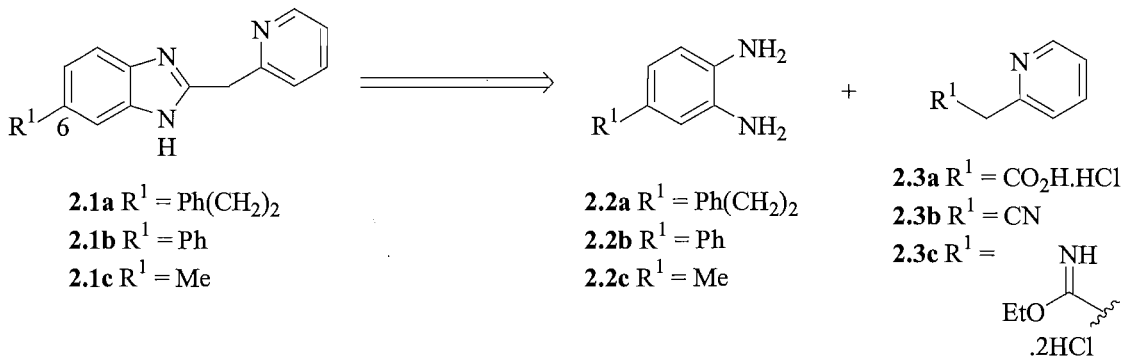


2.1c(1) **2.1c(2)**

Figure 2.3. Tautomeric forms of **2.1c**.

2.2 ATTEMPTED SYNTHESIS OF THE BENZIMIDAZOLES 2.1a-c

A retrosynthetic analysis of the proposed benzimidazole-based inhibitors **2.1a-c** suggested that the heterocyclic nucleus would be best obtained in the final synthetic step. Disconnection of **2.1a-c** afforded the diamines **2.2a-c**, which were to be reacted with appropriate derivatives (**2.3a-c**) that possess a pyridin-2-ylmethyl moiety. The following section details the synthesis of diamines **2.2a-b**, the attempted preparation of **2.1a-b**, and the difficulties encountered upon synthesis of **2.1c**.

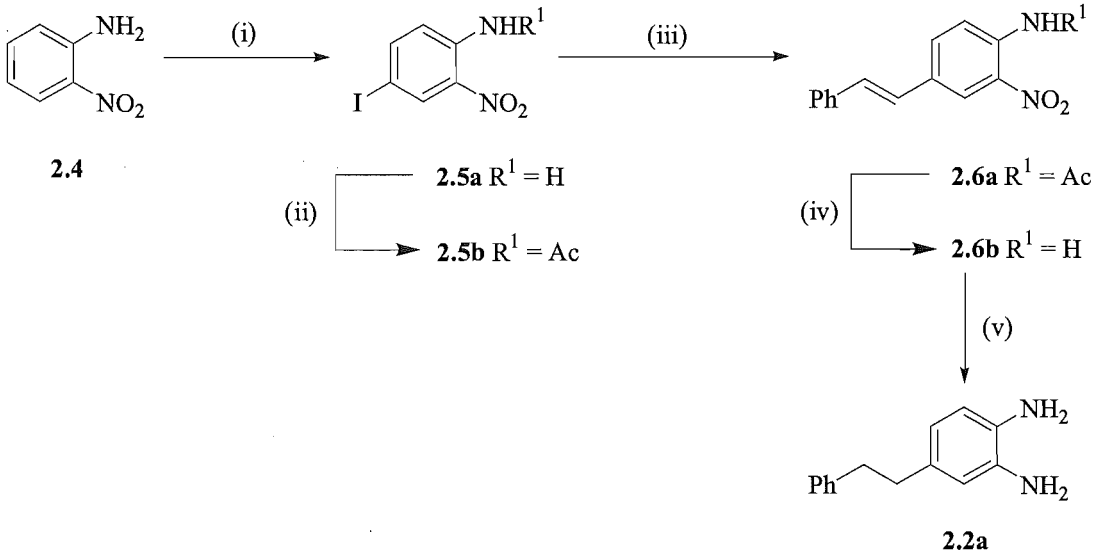


Scheme 2.1. Retrosynthetic analysis of the benzimidazoles **2.1a-c**.

2.2.1. Synthesis of the diamine intermediate 2.2a

The synthesis of bibenzyl diamine intermediate **2.2a** is illustrated in Scheme 2.2. 2-Nitroaniline (**2.4**) was iodinated with pyridine iodine monochloride[8] to give **2.5a** in 97% yield, and subsequent acylation with acetyl chloride afforded **2.5b** in 91% yield. Treatment of **2.5b** with styrene under Heck arylation conditions using palladium acetate as the cross-coupling catalyst[9] gave **2.6a** in 51% yield as pure *E* isomer. Assignment of **2.6a** as the *E* isomer was based on the relatively large coupling constant (J = 16.6 Hz) given by an AB quartet for the alkene proton resonance at δ 7.09 ppm. This observation is in agreement with the larger coupling constants expected for vicinal protons in 1,2-disubstituted *E* alkenes (commonly J = 14-16 Hz), in comparison to those obtained for vicinal protons in the corresponding *Z* alkenes (typically J = 6-8 Hz).[10] Deacetylation of

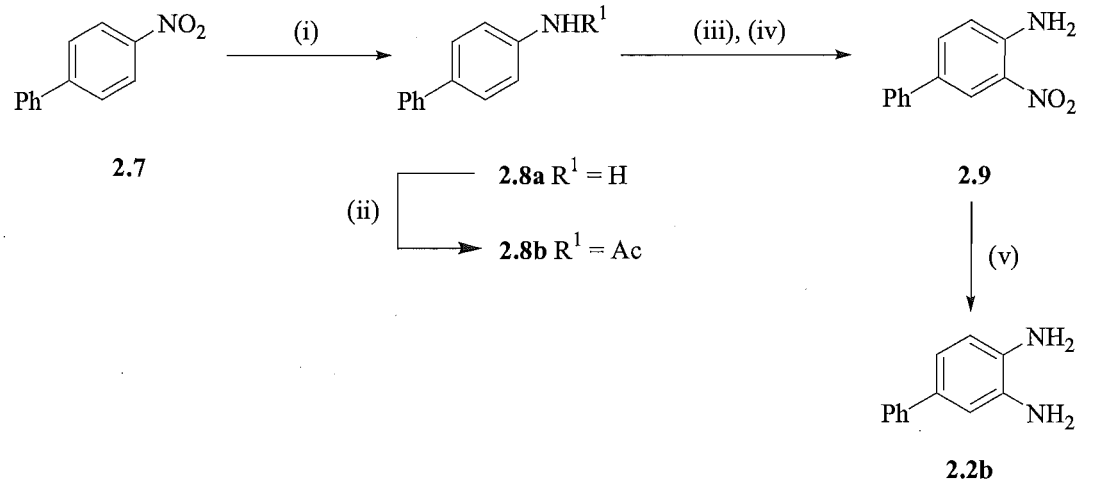
2.6a in 4:1 ethanol/5 M aqueous sodium hydroxide[11] gave **2.6b** quantitatively, and subsequent reduction with hydrogen over platinum oxide (Adams catalyst) afforded the desired diamine **2.2a** in 96% yield.



Scheme 2.2. *Reagents and conditions:* (i) PyrICl, 30% $AcOH_{(aq)}$, r.t., 30 min (97%). (ii) AcCl, reflux, 15 min (91%). (iii) Styrene, 10 mol % $Pd(OAc)_2$, 20 mol % (*o*-tol)$_3$P, Et_3N, MeCN, reflux, 3 h (51%). (iv) 4:1 EtOH/5 M $NaOH_{(aq)}$, reflux, 1 h (quantitative). (v) H_2, PtO_2, EtOH, r.t., 17 h (96%).

2.2.2. Synthesis of the diamine intermediate 2.2b

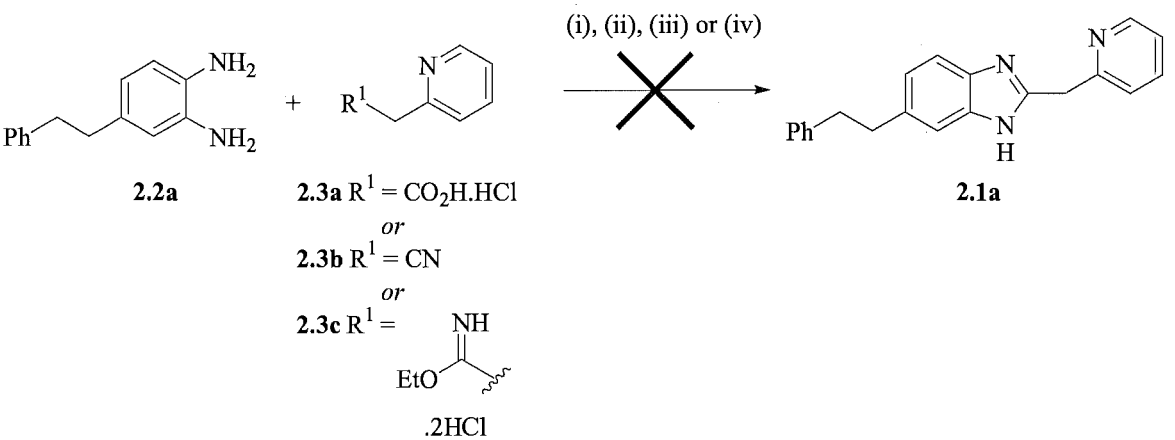
Preparation of the biphenyl diamine intermediate **2.2b** is illustrated in Scheme 2.3. 4-Nitro-1,1'-biphenyl (**2.7**) was reduced with hydrogen over 10% palladium on charcoal to afford the amine **2.8a** in 96% yield, and subsequent acylation with acetyl chloride gave **2.8b** in quantitative yield. Nitration of **2.8b** with nitric acid in acetic acid,[12] followed by deacetylation in 4:1 ethanol/5 M aqueous sodium hydroxide, gave **2.9** in 76% yield over two steps. Reduction of **2.9** with hydrogen over Adams catalyst afforded the desired diamine **2.2b** in 82% yield.



Scheme 2.3. *Reagents and conditions:* (i) H_2, 10% Pd/C, EtOH, r.t., 17 h (96%). (ii) AcCl, reflux, 15 min (quantitative). (iii) HNO_3, AcOH, 70°C, 1 h. (iv) 4:1 EtOH/5 M $NaOH_{(aq)}$, reflux, 1 h (76% over two steps). (v) H_2, PtO_2, EtOH, r.t., 6 h (82%).

2.2.3 Attempted synthesis of benzimidazoles 2.1a-b

The next stage involved determining the optimal conditions for preparation of the benzimidazoles **2.1a-c**. Scheme 2.4 illustrates that separate reaction of **2.2a** with **2.3a-c** did not afford the expected product **2.1a**.



Scheme 2.4. *Reagents and conditions:* (i) **2.3a**, PPA, 170-180°C, 5 h. (ii) **2.3a**, 4 M HCl, 120-130°C, 6 h. (iii) **2.3b**, PPA, 250°C, 4 h. (iv) **2.3c**, AcOH, reflux, 15 h.

Reaction of the diamine **2.2a** with 2-pyridylacetic acid hydrochloride (**2.3a**) in PPA at 170-180°C[13] gave an intractable mixture, and the presence of **2.1a** was not revealed by ^{1}H NMR spectroscopy. Analysis by reverse-phase HPLC illustrated that a complex mixture of compounds was generated by the reaction of **2.2a** with **2.3a** in PPA (Figure 2.4). The reaction of **2.2a** with **2.3a** in refluxing 4 M hydrochloric acid[14] again resulted in extensive byproduct formation as shown by ^{1}H NMR spectroscopy. Reaction of **2.2a** with 2-pyridylacetonitrile (**2.3b**) in PPA at 250°C[15] also gave an intractable mixture of compounds by ^{1}H NMR spectroscopy. At this stage milder methods for the preparation of benzimidazoles were sought, and one such procedure involves reaction of an aromatic diamine with an imidate.[16] To this end, nitrile **2.3b** was dissolved in 10:1 benzene/ethanol at 0°C, then saturated with hydrogen chloride gas, and the resulting suspension was stirred at r.t. for 16 h. After isolation, the imidate **2.3c** was immediately reacted with **2.2a** in refluxing acetic acid,[17] but once again an intractable mixture of byproducts was produced. The ^{1}H NMR spectrum of this mixture illustrated that little or no **2.1a** was formed, as it did

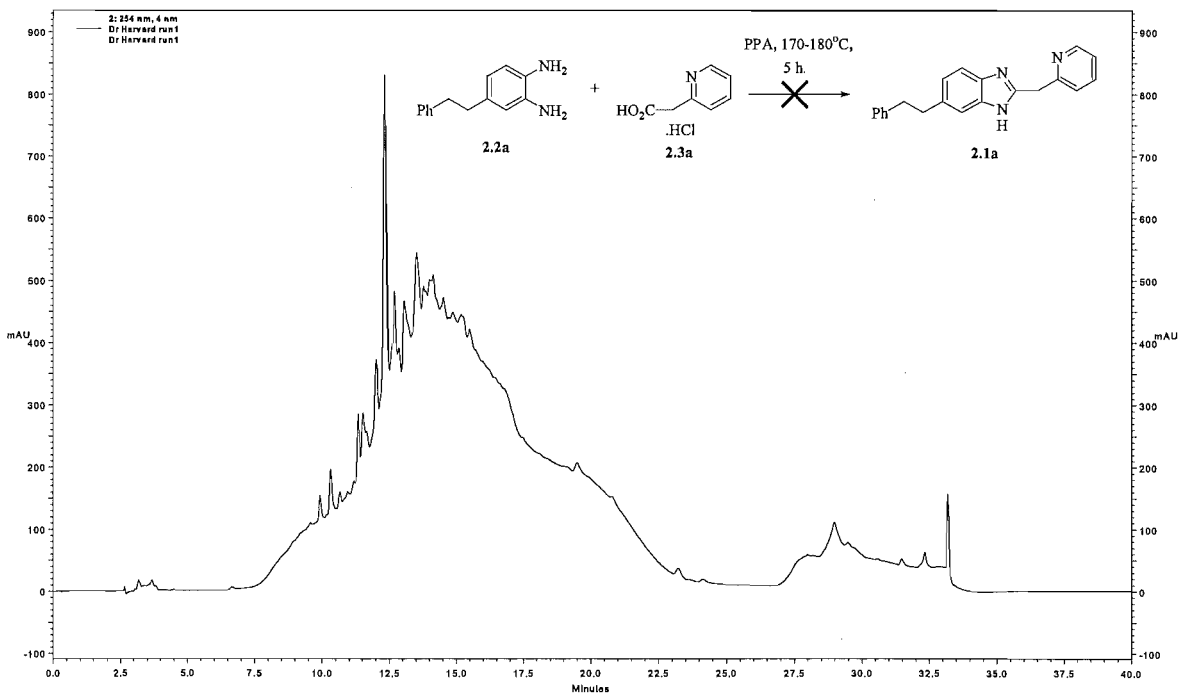
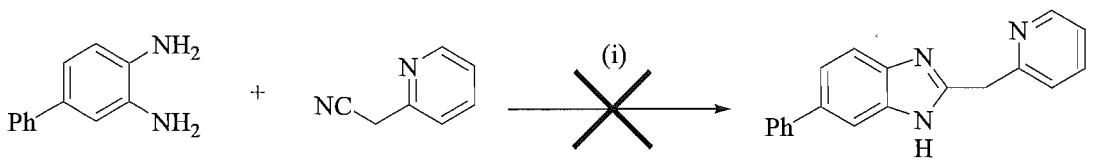


Figure 2.4. HPLC trace of the mixture obtained from the reaction of the diamine **2.2a** and **2.3a** in PPA at 170-180°C in the attempted formation of **2.1a**.

not contain the resonances expected for the downfield pyridyl protons (δ approximately 8.8-8.3 ppm) or the methylene group (δ approximately 4.4 ppm).

Diamine **2.2b** was reacted with **2.3b** in PPA at 250°C in an attempt to prepare **2.2b** (Scheme 2.5), however ^{1}H NMR spectroscopy revealed that a wide range of byproducts had been formed.



2.2b **2.3b** **2.1b**

Scheme 2.5. *Reagents and conditions:* (i) PPA, 250°C, 4 h.

Reverse-phase HPLC analysis of the mixture generated by reaction of **2.2b** and **2.3b** (Figure 2.5) illustrated that less byproducts were formed than in the reaction of **2.2a** and

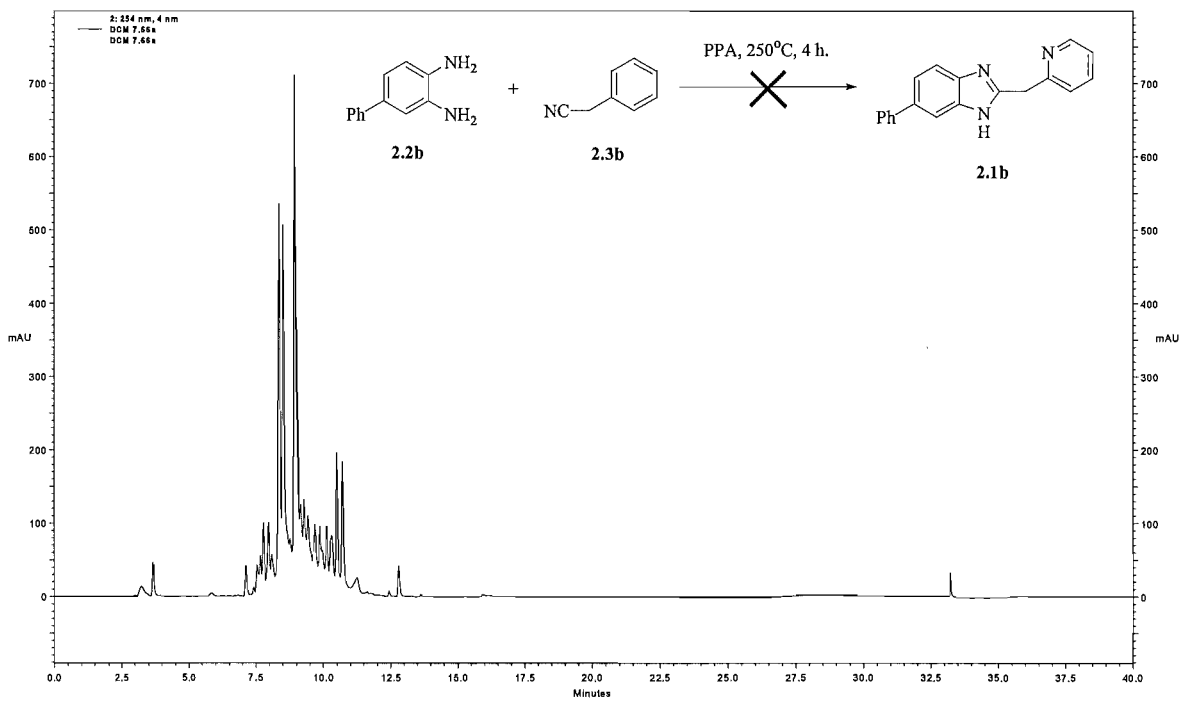


Figure 2.5: HPLC trace of the mixture obtained from the reaction of **2.2a** and **2.3b** in PPA at 250°C in the attempted preparation of **2.1b**.

2.3a (see Figure 2.4). However, the level of byproduct formation was sufficient to eliminate this methodology from any subsequent attempts toward the preparation of **2.1b**.

The cause of excessive byproduct formation in reactions which utilised **2.2a** or **2.2b** was postulated to result from instability of the diamine starting materials under the relatively harsh reaction conditions utilised in the attempted preparation of **2.1a-b**. Precedent exists for the degradation of bibenzyl (**2.10**, Figure 2.6), the basic carbon backbone of **2.2a**, to a range of byproducts.[18] The carbon-carbon bond strength of the adjacent methylene groups in **2.10** (marked by a dotted line in Figure 2.6) was calculated to be 235 kJ mol^{-1},[18e] therefore bond cleavage requires comparatively little energy. It was envisaged that **2.2a**, with its bibenzyl motif, would undergo cleavage under the harsh conditions used in the attempted preparation of **2.1a**. Degradation would give the range of byproducts observed in both the ^{1}H NMR spectra and HPLC traces. The bond strength between the two phenyl rings of **2.2b** would be considerably higher (calculated as 480 kJ mol^{-1} for its simple biphenyl analogue **2.11**,[18e] Figure 2.6) than that between the adjacent methylene groups in **2.2a**. This accounted for the lower level of byproduct formation observed in the HPLC trace derived from the attempted synthesis of **2.1b** (see Figure 2.5). The following section

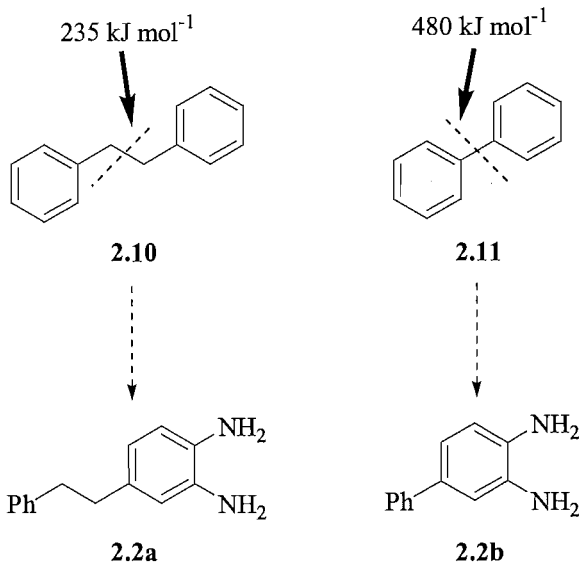
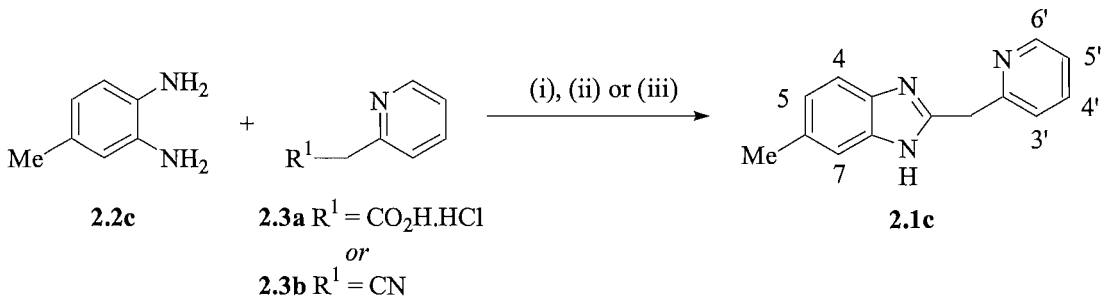


Figure 2.6. Structures and calculated energies required for bond cleavage of bibenzyl (**2.10**) and biphenyl (**2.11**), the basic carbon backbones of **2.2a** and **2.2b** respectively. The points of cleavage are represented by dashed lines.

details the reaction of **2.2c** (R^1 = Me), under analogous conditions to those used in the attempted synthesis of **2.1a-b**, to test the proposition that this diamine was more stable than **2.2a-b** (R^1 = aromatic).

2.2.4 Synthesis and reactivity at the methylene bridge of benzimidazole 2.1c

Separate reaction of **2.2c** with **2.3a** or **2.3b** in PPA at 170-180°C afforded the desired benzimidazole **2.1c** in yields of 50% (purified) and 78% (crude mass return) respectively (Scheme 2.6). Purification was not performed on the crude product obtained from the reaction of **2.2c** and **2.3b**, due to the sufficient amount of pure **2.1c** isolated from the reaction of **2.2c** and **2.3a**. The numbering system for **2.1c** is included in Scheme 2.6. Diamine **2.2c** was also reacted with **2.3a** in refluxing 4 M hydrochloric acid to give crude **2.1c** as the dihydrochloride salt with a quantitative return of mass. However, this compound degraded upon standing. Overall, **2.2c** appeared to be a more stable reactant for benzimidazole preparation than its analogues **2.2a-b**, which both possessed aromatic R^1 groups.



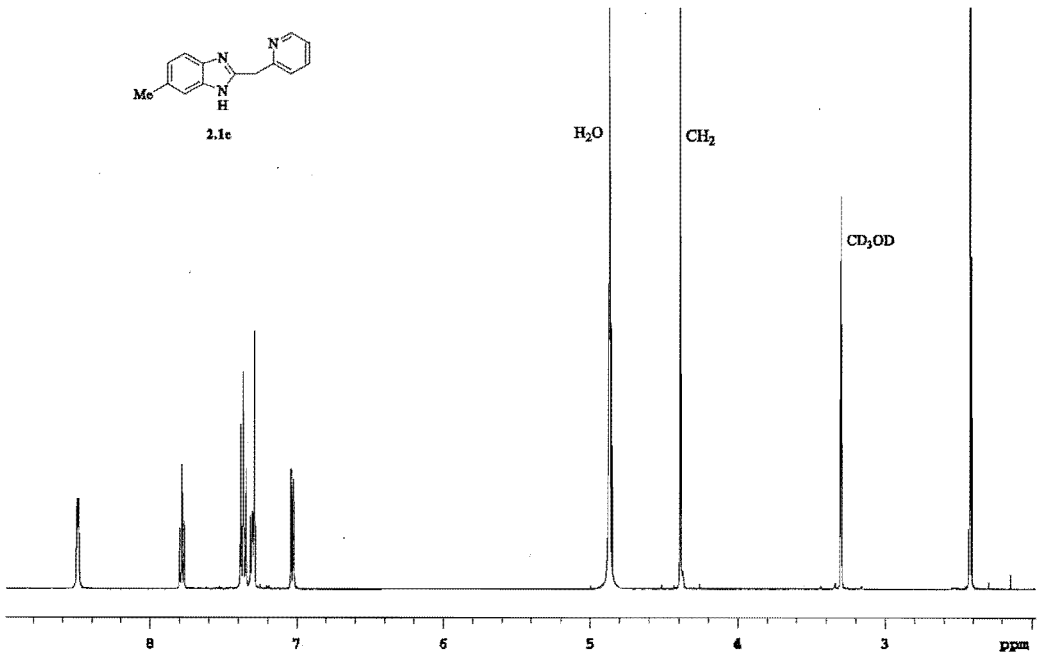
2.2c **2.3a** $R^1 = CO_2H.HCl$ *or* **2.3b** $R^1 = CN$ **2.1c**

Scheme 2.6. *Reagents and conditions:* (i) **2.3a**, PPA, 170-180°C, 5 h (50%). (ii) **2.3b**, PPA, 170-180°C, 5 h (78% crude mass return). (iii) **2.3a**, 4 M HCl, 120-130°C, 6 h (quantitative mass return).

A noticeable change was observed in the ^{1}H NMR spectrum of **2.1c** upon standing in CD_3OD. Figure 2.7a displays the ^{1}H NMR spectrum of **2.1c** at 0 h, with the resonance at δ 4.38 ppm assigned to the methylene group. However, after 16 h this resonance was no longer present (Figure 2.7b). A ^{2}H NMR spectrum of the derivatised **2.1c** in CH_3CN, with

one drop of $CDCl_3$ as a reference solvent, gave a weak resonance at δ 4.02 ppm, which was postulated to result from deuteration of the methylene group.

(a)



(b)

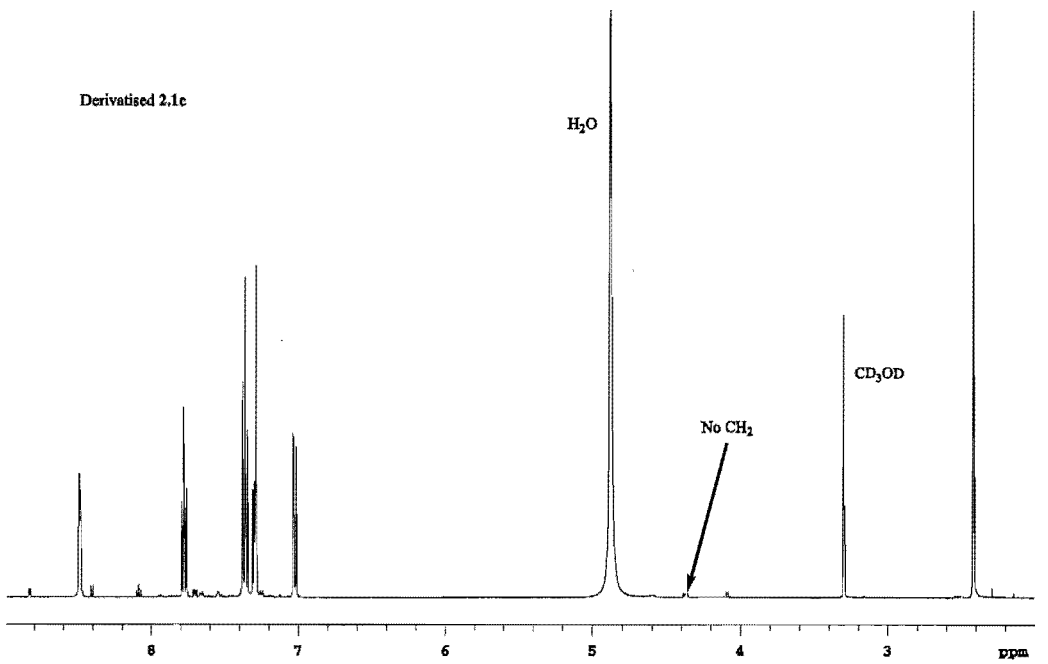
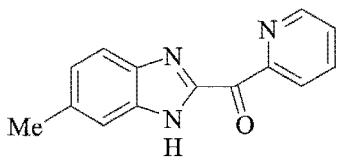


Figure 2.7. ^{1}H NMR spectra of **2.1c** in CD_3OD after 0 h (a) and 16 h (b).

Analysis of derivatised **2.1c** by electrospray ionisation LRMS gave molecular ions corresponding to the protonated equivalents of native **2.1c**, mono- and dideuterated **2.1c**, and the ketone **2.12** (Figure 2.8). These results revealed that the reactivity of the methylene bridge possessed by **2.1c** allowed a combination of deuteration and oxidation to occur.



2.12

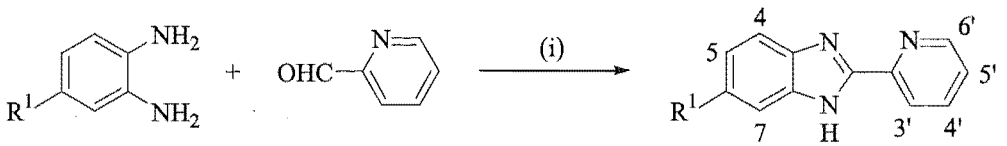
Figure 2.8. Ketone **2.12**, derived from **2.1c**.

Due to the inability to effect formation of **2.1a-b**, and the potential problems associated with the reactivity of the methylene bridge in compounds analogous to **2.1c**, further synthetic work in this area was discontinued. An alternative preparative route to benzimidazoles with a slightly modified structure was undertaken.

2.3 SYNTHESIS AND ASSAY OF BENZIMIDAZOLES 2.14a-c

A milder synthetic method for the preparation of benzimidazoles was sought to overcome the difficulties encountered in the previous section towards the synthesis of **2.1a-b**. The reported synthesis of a range of benzimidazoles was effected by the reaction of aromatic diamines with aryl aldehydes in nitrobenzene at 145°C for 18 h.[19] Nitrobenzene acted as both the solvent and an oxidant to complete benzimidazole formation. Application of the nitrobenzene-based benzimidazole preparation gave **2.14a-c** by the synthetic route displayed in Scheme 2.7. Reaction of the diamines **2.2a-c** with 2-pyridinecarboxaldehyde (**2.13**) in nitrobenzene at 150°C afforded **2.14a-c** in yields of 43%, 57% and 46% respectively. The numbering system for compounds of type **2.14** is included in Scheme

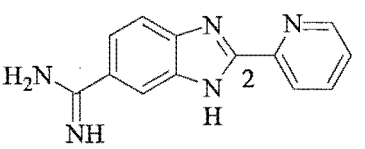
2.7. This synthetic methodology was considered advantageous since these compounds lacked the methylene bridge that was prone to the deuteration/oxidation observed for **2.1c**. An analogous compound (**1.13e**, highlighted in Scheme 2.7), with a pyridin-2-yl moiety at C2, displayed a 5-fold enhancement for trypsin inhibition when Zn^{2+} was present.[5]



2.2a $R^1 = Ph(CH_2)_2$
2.2b $R^1 = Ph$
2.2c $R^1 = Me$

2.13

2.14a $R^1 = Ph(CH_2)_2$
2.14b $R^1 = Ph$
2.14c $R^1 = Me$



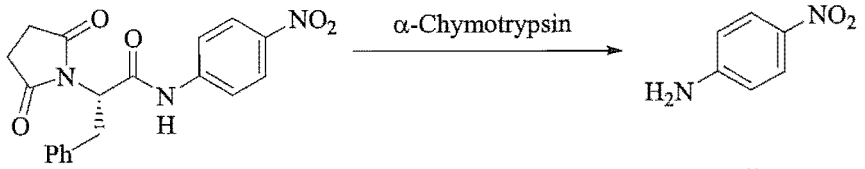
1.13e

Trypsin K_i (ETDA) = 101 μM
K_i (Zn^{2+}) = 20.9 μM
Zn^{2+} factor = 5

Scheme 2.7. (i) $PhNO_2$, 150°C, 18 h (**2.14a**, 43%), (**2.14b**, 57%), (**2.13c**, 46%).

2.3.1 Assay results for the α-chymotrypsin inhibitors 2.14a-c

The benzimidazoles **2.14a-c** were tested for inhibitory activity against α-chymotrypsin using the established method of Cannell *et al.*[20] This assay is reliant on a colourimetric technique, whereby enzymatic activity is measured by the cleavage of colourless *N*-succinyl-L-phenylalanine-4-nitroanilide **2.15** to yellow-coloured 4-nitroaniline **2.16** (Scheme 2.8). The level of **2.16** released is colourimetrically detected by the level of absorbed light at a wavelength of 405 nm.



Colourless
2.15

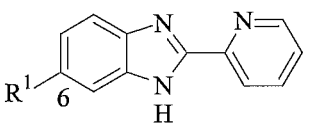
Yellow
2.16

Scheme 2.8. Enzymatic breakdown of **2.15** to give the coloured product **2.16**.

Solutions of the benzimidazoles **2.14a-c** were made up to 10 mM in distilled methanol and diluted by a factor of 10 sequentially six times, *i.e.* a range of seven concentrations (10 mM, 1.0 mM, 0.1 mM, 10 μM, 1.0 μM, 0.1 μM, and 10 nM) at which inhibition was tested. Determination of the inhibition elicited by the test compounds in the presence of Zn^{2+} is as follows. The inhibitor test solution, Tris-HCl buffer (pH 7.6), a solution of α-chymotrypsin in Tris-HCl buffer (pH 7.6) and a solution of 1.0 mM zinc chloride in distilled water were added to the appropriate wells of a 96 well assay plate. The zinc chloride solution was added to determine the level of α-chymotrypsin inhibition elicited by **2.14a-c** via the proposed ternary complex between the enzyme, Zn^{2+} and inhibitor. After incubation at 37°C for 30 min, the substrate **2.15** in Tris-HCl buffer (pH 7.6) was added, and the assay well plates were returned to the incubator. Upon substrate addition, the final concentration of Zn^{2+} was 0.17 mM. The absorbance was read at 405 nm every hour until the maximum readings exceeded 1 absorbance unit. The calculated percent inhibition values are a measure of the enzyme activity relative to that when no enzyme was present (no substrate turnover, 100% "inhibition") and when no inhibitor was present (full substrate turnover, 0% inhibition). To this end, control samples, with Tris-HCl buffer (pH 7.6) in place of the α-chymotrypsin solution, were included at all inhibitor concentrations to determine the level of absorbance when there was no turnover of **2.15**. Maximum substrate turnover was calculated by the average absorbance across six wells where distilled methanol replaced inhibitor solution. Each inhibitor concentration was tested in triplicate and average absorbances were used to calculate the levels of inhibition. The potency of each compound was measured by its IC_{50} value - this is the concentration of inhibitor required for half-maximal inhibition of the target enzyme.[21] The IC_{50} values were

calculated by plotting percent inhibition (y axis) against $-\log_{10}$(inhibitor concentration) (x axis) in Microsoft Excel®.

Activity of the test compounds in the presence of EDTA was performed in an analogous manner, except that the Zn^{2+} solution was replaced by 5.0 mM EDTA disodium dihydrate in distilled water. Upon substrate addition, the final concentration of EDTA was 0.83 mM. Addition of the EDTA solution would determine the level of inhibition displayed by **2.14a-c** without the aid of any metal ions. Comparison of the activities elicited by **2.14a-c** in Zn^{2+} or ETDA would resolve whether the activity of the proposed inhibitors against α-chymotrypsin was enhanced due to the presence of metal ions. Results for the assay of **2.14a-c** against α-chymotrypsin are presented in Table 2.1.



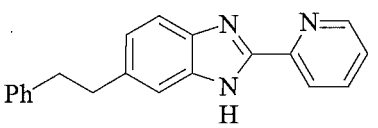
No.	R^1	IC_{50} (μM)	
		Zn^{2+}	EDTA
2.14a	$Ph(CH_2)_2$	81	>17
2.14b	Ph	>17	>17
2.14c	Me	>167	681

Table 2.1. IC_{50} values for **2.14a-c** in the presence of Zn^{2+} or EDTA.

Table 2.1 illustrates that **2.14a-c** were moderate to weak inhibitors of α-chymotrypsin. An exact IC_{50} value was not obtained for **2.14a** (in the presence of EDTA), **2.14b** (in the presence of Zn^{2+} or EDTA), and **2.14c** (in the presence of Zn^{2+}), due to precipitation of the inhibitor in the assay wells. Typically this occurred at the two or three highest concentrations of inhibitor. Less than half-maximal enzyme inhibition was achieved at the inhibitor concentrations where precipitation did not occur, hence an IC_{50} value could not be calculated. Benzimidazole **2.14a** displayed moderate inhibition of α-chymotrypsin, with

an IC_{50} value of 81 μM in the presence of Zn^{2+}. No quantifiable increase in inhibition attributable to enhanced Zn^{2+}-mediated binding was determined for **2.14a**, as an IC_{50} value could not be determined for the inhibitor when EDTA was present. Compound **2.14b** was an extremely poor inhibitor, showing minimal effect on enzyme activity when precipitation of the inhibitor did not occur in the wells. Benzimidazole **2.14c** was a poor inhibitor when EDTA was present in solution (IC_{50} = 681 μM), and the opposite trend to that for **2.14a** was displayed, whereby no IC_{50} value could be calculated for the solution containing Zn^{2+}. The low inhibitory activity displayed by **2.14c** was expected due to the absence of an aromatic R^1 moiety at C6.

A more detailed examination of the inhibition elicited by **2.14a** against α-chymotrypsin is presented in Table 2.2. Overall, the data illustrates that no Zn^{2+}-mediated enhancement of



2.14a

Conc. of **2.14a** in assay well (M)	% inhibition		(1) – (2) (%)
	Zn^{2+} present (1)	EDTA present (2)	
1.67×10^{-3}	-*	-*	-#
1.67×10^{-4}	73	-*	-#
1.67×10^{-5}	0	6	-6
1.67×10^{-6}	5	12	-7
1.67×10^{-7}	0	4	-4
1.67×10^{-8}	11	1	10
1.67×10^{-9}	5	0	5

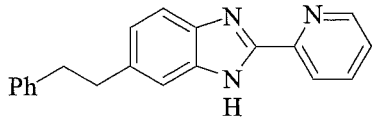
* = precipitation occurred in reaction wells during assay.
# = (1) – (2) could not be calculated due to the occurrance of inhibitor precipitation during the assay.

Table 2.2. Inhibition data for all concentrations at which **2.14a** was assayed for inhibition of α-chymotrypsin in the presence of Zn^{2+} or EDTA.

inhibition was observed at any inhibitor concentration. This differed from the enhancement reported by Katz, *et al*[5] whereby the addition of Zn^{2+} increased the potency of an inhibitor series towards selected serine proteases (see Chapter One, Section 1.2.1). A lack of enhancement for the inhibition of α-chymotrypsin by **2.14a** in the presence of Zn^{2+} compared to when EDTA was present suggested that inhibition was not occurring in the fashion proposed in Figure 2.2. Instead, inhibition is likely the result of uncomplexed **2.14a**.

2.4 SYNTHESIS AND ASSAY OF THE HYDROCHLORIDE SALT OF 2.14a

The hydrochloride salt of **2.14a** was prepared to determine whether an improvement in solubility, in comparison to the free base **2.14a**, would allow for IC_{50} values to be calculated in the presence of Zn^{2+} and EDTA. As a result, any Zn^{2+}-dependent enhancement in the inhibition of α-chymotrypsin at higher concentrations of **2.14a** would be revealed. To this end, **2.14a** was dissolved in ethyl acetate, and hydrochloride gas passed through the solution until a solid formed. Removal of the solvent afforded the dihydrochloride salt of **2.14a** in 84% yield (see Experimental for details). This compound was assayed under the same conditions used for **2.14a-c**. The assay result is displayed in Table 2.3, and the inhibitory activity of the free base of **2.14** is included for comparison.



2.14a

No.	IC_{50} (μM)	
	Zn^{2+}	EDTA
2.14a	81	>17
2.14a.2HCl	82	>17

Table 2.3. IC_{50} values for **2.14a.2HCl** in the presence of Zn^{2+} or EDTA. The IC_{50} values for **2.14a** are shown for comparison.

Preparation of the dihydrochloride salt of **2.14a** gave no increase in activity against α-chymotrypsin, or an improvement in its solubility properties. An almost identical inhibitory profile to that of the free base **2.14a** was observed, with an IC_{50} value of 82 μM determined for the inhibitor in the presence of Zn^{2+}, and no exact IC_{50} value was calculated when EDTA was present due to the occurrence of precipitation in the assay wells. It was postulated that the dihydrochloride salt was immediately converted to its free base in the neutral pH environment of an assay well. Therefore, no change in the solubility of **2.14a** was observed. The reported pK_a values for a range of benzimidazoles (~5-7)[22] and pyridine (5.36)[23] are such that deprotonation of **2.14a** dihydrochloride to the free base would occur in the neutral pH (7.6) present in the assay wells.

2.5 DETERMINATION OF THE ABILITY OF 2.14b TO ACT AS A LIGAND FOR TRANSITION METALS

A potential factor that would contribute to the lack of Zn^{2+}-enhanced enzyme inhibition observed for **2.14a-c** is that the 2-(pyridin-2-yl)benzimidazole-based scaffold was not able to form a complex with the metal ion. The ability of **2.14b** to act as a ligand was determined by ^1H NMR spectroscopy. Initially, this compound was dissolved in CD_3OD, and the ^1H NMR spectrum that was recorded is displayed in Figure 2.9.

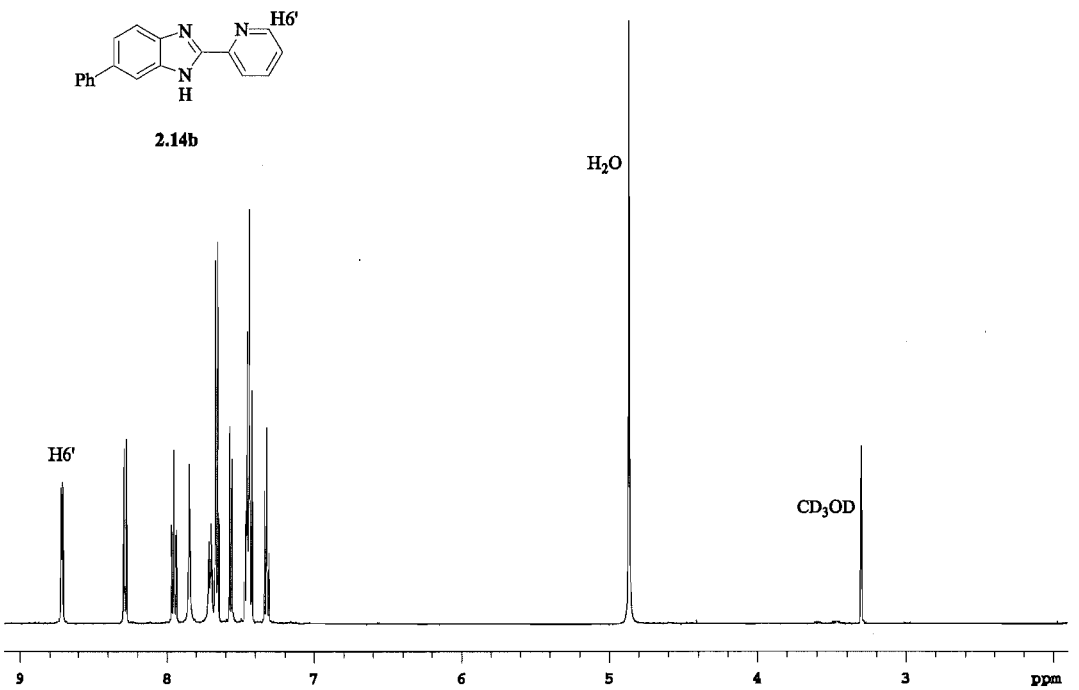


Figure 2.9. ^1H NMR spectrum of **2.14b** in CD_3OD.

One equivalent of zinc diacetate ($Zn(OAc)_2$) was added to the solution of **2.14b** in CD_3OD, and noticeable changes were observed in the chemical shifts of the ^1H NMR spectrum. Figure 2.10 illustrates that line broadening of the signals in the aromatic region (δ approximately 7.2-8.6 ppm) occurred. Line broadening of this type is typically observed upon complexation due to non-rigidity and ligand exchange in the complex,[24] and it has been observed for the Zn^{2+} complexes of organic molecules.[25] Use of deuterated NMR solvents other than CD_3OD gave no improvement in the spectrum, as the level of line

broadening was approximately the same, or the complex was insoluble and no ^{1}H NMR spectrum could be obtained. The positions of signals for equivalent protons in the two spectra were noticeably different. In Figure 2.9, the resonance at δ 8.72 ppm corresponded to H6' of the pyridyl ring, whereas in Figure 2.10 the most downfield signal resonated at δ 8.57 ppm, which was determined to be H6' in the COSY spectrum. A shift of this magnitude was expected as H6', of all the protons present in **2.14b**, is closest to a nitrogen involved in the formation of a dative bond with the metal.

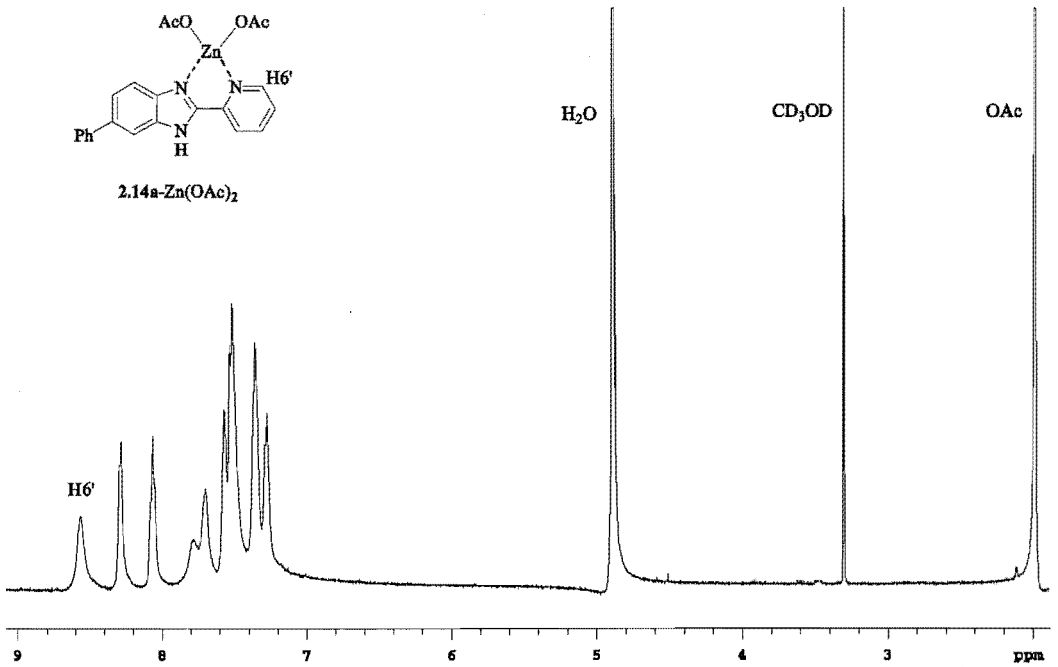


Figure 2.10. ^{1}H NMR spectrum of the complex formed between **2.14b** and $Zn(OAc)_2$ in CD_3OD.

Attempted formation of a crystal suitable for X-ray crystallography from **2.14b** and $Zn(OAc)_2$ was unsuccessful. Techniques used for crystal preparation included vapour diffusion (methanol/ether, DMSO/benzene, methanol/water and methanol/acetonitrile), and evaporation of the solvent (methanol, methanol/water and methanol/acetonitrile) from **2.14b**. A further attempt toward crystal growth, by the evaporation of methanol from a solution of **2.14b** and $Cu(NO_3)_2$, was also ineffective.

2.5 CONCLUSION AND FUTURE WORK

Potent Zn^{2+}-enhanced inhibition of trypsin-like serine proteases has been reported by a series of benzimidazole-based compounds.[5] This novel binding mode was applied to the development of novel α-chymotrypsin inhibitors. Initial efforts were focused on the synthesis of the proposed inhibitors **2.1a-b**, which featured a 2-(pyridin-2-ylmethyl)benzimidazole scaffold upon which aromatic recognition elements were attached at the C6 position (denoted R^1). The aromatic groups were considered essential for recognition of the putative inhibitors by α-chymotrypsin, due to the hydrophobic S_1 subsite possessed by this enzyme. However, preparation of the benzimidazoles **2.1a-b** by a variety of methods was unsuccessful. The diamine precursors **2.2a-b** were postulated to be unstable under the reaction conditions in which benzimidazole formation was attempted. Preparation of **2.1c**, which differed from **2.1a-b** by the presence of a methyl group at R^1, demonstrated the stability of the diamine **2.2c** (R^1 = Me) in comparison to **2.2a-b** (R^1 = aromatic). However, the desired product **2.1c** was prone to deuteration and oxidation at the methylene group. As a result, the structural integrity of inhibitors based on structure **2.1** was compromised due to the potential for unwanted side reactions at this position.

Subsequently, the synthesis of a slightly modified benzimidazole scaffold was undertaken, and these structures were based on a 2-(pyridin-2-yl)benzimidazole motif. This work identified a relatively mild synthetic step for the synthesis of the benzimidazoles **2.14a-c**, which did not lead to the excessive formation of byproducts observed in the attempted synthesis of **2.1a-b**. Benzimidazoles **2.14a-b** were moderate to weak inhibitors of α-chymotrypsin, despite the presence of aromatic moieties at R^1 that were expected to act as suitable recognition elements for the enzyme. Compound **2.14c**, with a methyl R^1 group, was a poor inhibitor as expected due to the lack of an aromatic functionality at this position. No Zn^{2+}-enhanced inhibition of α-chymotrypsin was exhibited by **2.14a-c** due to the poor solubility profile displayed by these compounds in the assay conditions used. A closer examination of the inhibition elicited by **2.14a** gave no evidence of enhanced α-chymotrypsin inhibition in the presence of Zn^{2+} at any inhibitor concentration. An attempted improvement of the solubility displayed by **2.14a** involved the formation of its

dihydrochloride salt, but no change in the inhibitory activity or solubility profile was observed. Overall, a Zn^{2+}-enhanced mode of inhibition toward α-chymotrypsin was not elicited by the 2-(pyridin-2-yl)benzimidazole scaffold. However, it is uncertain whether this was a result of the scaffold used, inappropriate groups at R^1, or the properties of the enzyme active site itself.

The proposed binding mode of **2.14a-c** required that the 2-(pyridin-2-yl)benzimidazole scaffold acted as a ligand to Zn^{2+}. Addition of $Zn(OAc)_2$ to **2.14b** in CD_3OD caused line broadening and signal shifts to occur in the ^1H NMR spectrum, illustrating that complex formation was taking place. However, no crystal suitable for X-ray crystallography was obtained with either Zn^{2+} or Cu^{2+} as the metal centre.

Future work in this area could involve appending more polar groups to certain positions of the benzimidazole scaffold to improve its solubility, *e.g.* hydroxyl groups on the pyridyl ring. Additionally, a thorough investigation of the optimal group at R^1 for eliciting inhibition of α-chymotrypsin could be undertaken.

2.6 REFERENCES FOR CHAPTER TWO

1. (a) Henderson, R.; Wright, C. S.; Hess, G. P.; Blow, D. M. *Cold Spring Harbor Symposia on Quantitative Biology* **1971**, *36*, 63. (b) Sigler, P. B.; Blow, D. M.; Matthews, B. W.; Henderson, R. *J. Mol. Biol.* **1968**, *35*, 143. (c) Matthews, B. W.; Sigler, P. B.; Henderson, R.; Blow, D. M. *Nature* **1967**, *214*, 652.

2. (a) Hedstrom, L. *Chem. Rev.* **2002**, *102*, 4501. (b) Perona, J. J.; Craik, C. S. *Prot. Sci.* **1995**, *4*, 337. (c) Kashima, A.; Inoue, Y.; Sugio, S.; Maeda, I.; Nose, T.; Shimohigashi, Y. *Eur. J. Biochem.* **1998**, *255*, 12.

3. Pochet, L.; Doucet, C.; Dive, G.; Wouters, J.; Masareel, B.; Reboud-Ravaux, M.; Pirotte, B. *Bioorg. Med. Chem.* **2000**, *8*, 1489.

4. (a) Janoff, A. *Ann. Rev. Med.* **1985**, *36*, 207. (b) Janoff, A. *Am. Rev. Respir. Dis.* **1985**, *132*, 417. (c) Snider, G. L. *Drug Dev. Res.* **1987**, *10*, 235. (d) Nadel, J. A. *Am. Rev. Respir. Dis.* **1991**, *14*, S48. (e) Demling, R. H. *Annu. Rev. Med.* **1995**, *46*, 193.

5. Katz, B. A.; Clark, J. M.; Finer-Moore, J. S.; Jenkins, T. E.; Johnson, C. R.; Ross, M. J.; Luong, C.; Moore, W. R.; Stroud, R. M. *Nature* **1998**, *391*, 608.

6. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.

7. Wallace, R. A.; Kurtz, A. N.; Niemann, C. *Biochemistry* **1963**, *2*, 824.

8. Muathen, H. A. *J. Chem. Res. (M)* **1994**, 2201.

9. Lee, S. H.; Cho, Y. J.; Bae, J. W.; Yoon, C. M. *Synth. Commun.* **2000**, *30*, 1003.

10. Kemp, W. *Organic Spectroscopy*; 3rd ed.; MacMillan Press Ltd: Basingstoke, 1991.

11. Budesinsky, Z.; Sluka, J.; Novak, J.; Danek, J. *Coll. Czech. Chem. Commun.* **1975**, *40*, 1089.

12. Campbell, N.; Anderson, W.; Gilmore, J. *J. Chem. Soc.* **1940**, 446.

13. Alcalde, E.; Dinares, I.; Perez-Garcia, L.; Roca, T. *Synthesis* **1992**, 395.

14. Krati, N.; Roizard, D.; Brembilla, A.; Lochon, P. *Bull. Chim. Soc. Fr.* **1989**, 443.

15. Hein, D. W.; Alheim, R. J.; Leavitt, J. J. *J. Am. Chem. Soc.* **1957**, *79*, 427.

16. Preston, P. N. *Chem. Rev.* **1974**, *74*, 279.

17. Hori, Y.; Bichenkova, E. V.; Wilton, A. N.; El-Attug, M. N.; Sadat-Ebrahimi, S.; Tanaka, T.; Kikuchi, Y.; Araki, M.; Sugiura, Y.; Douglas, K. T. *Biochemistry* **2001**, *40*, 603.

18. (a) Albini, A.; Spreti, S. *J. Chem. Soc., Perkin Trans. 2* **1987**, 1175. (b) Chiba, K.; Tagaya, H.; Yamauchi, T.; Thukahara, Y. *Chem. Lett.* **1985**, 945. (c) Stenberg, V. I.; Hei, R. D. *J. Org. Chem.* **1985**, *50*, 1810. (d) Burr, J. G.; Javeri, I. *Fuel* **1984**, *63*, 854. (e) Vernon, L. W. *Fuel* **1980**, *59*, 102.

19. Rangarajan, M.; Kim, J. S.; Sim, S.-P.; Liu, A.; Liu, L. F.; LaVoie, E. J. *Bioorg. Med. Chem.* **2000**, *8*, 2591.

20. Cannell, R. J. P.; Kellam, S. J.; Owsianka, A. M.; Walker, J. M. *Planta Med.* **1988**, *54*, 10.

21. Keleti, T. In *Basic Enzyme Kinetics*; Akademiai Kiado: Budapest, 1986, pp. 125-402.

22. Hofmann, K. In *Imidazole and Its Derivatives*; Interscience Publishers, Inc.: New York, 1953; Vol. 6, pp. 247-324.

23. Barnes, R. A. In *Pyridine and Its Derivatives*; Klingsberg, E., Ed.; Interscience Publishers, Inc.: New York, 1960; Vol. 14, pp. 1-97.

24. Crabtree, R. H. In *The Organometallic Chemistry of the Transition Metals*; 3rd ed.; John Wiley & Sons, Inc.: New York, 2001, pp. 259-291.

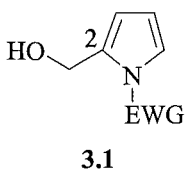
25. Froystein, N. A.; Sletten, E. *Acta Chem. Scand.* **1991**, *45*, 219.

CHAPTER THREE

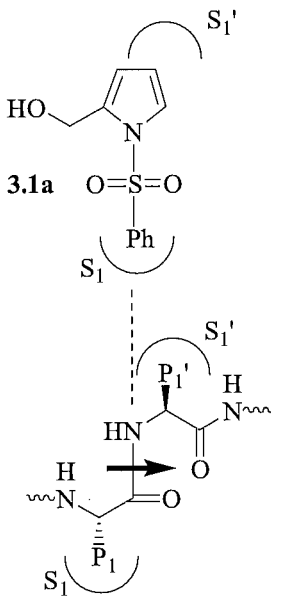
DEVELOPMENT OF C5-(HYDROXYALKYL)PYRROLE-BASED α-CHYMOTRYPSIN INHIBITORS

3.1 INTRODUCTION

The inhibition of α-chymotrypsin, a member of the serine protease family of enzymes,[1] is regarded as an important step in the development of inhibitors that target chymotrypsin-like enzymes.[2] Examples of this subclass of serine proteases include cathepsin G, mast cell chymase and human leukocyte elastase, and over-activity of these enzymes is implicated in a number of debilitating diseases in the human body.[3] (See Chapter One, Section 1.2 for further discussion of the disease states arising from serine protease over-activity.) α-Chymotrypsin is considered an excellent model system for serine protease inhibition due to its well-defined active site structure[4] and substrate specificity.[5] Previous work in these laboratories[6] revealed a series of *N*-substituted 2-(hydroxymethyl)pyrroles based on the general structure **3.1** (Figure 3.1) as a new class of α-chymotrypsin inhibitors. Compounds of type **3.1** were distinguished by the presence of an electron-withdrawing group (EWG) attached to the pyrrole ring nitrogen, and a hydroxymethyl group at the C2 position. Optimal inhibition for the range of analogues assayed was displayed by **3.1a**, and its pro-



3.1

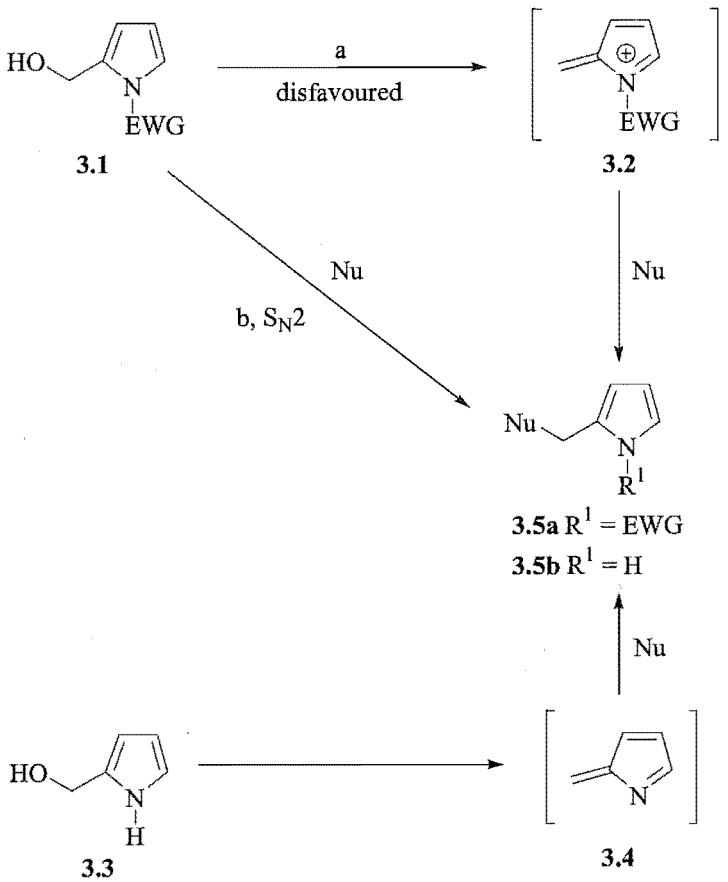


3.1a

Figure 3.1. General structure **3.1** of the *N*-substituted 2-(hydroxymethyl)pyrrole-based α-chymotrypsin inhibitors. The optimal inhibitor **3.1a** is shown, and its binding in the enzyme active site is correlated to that of the natural substrate (cleavage point indicated by a bold arrow).

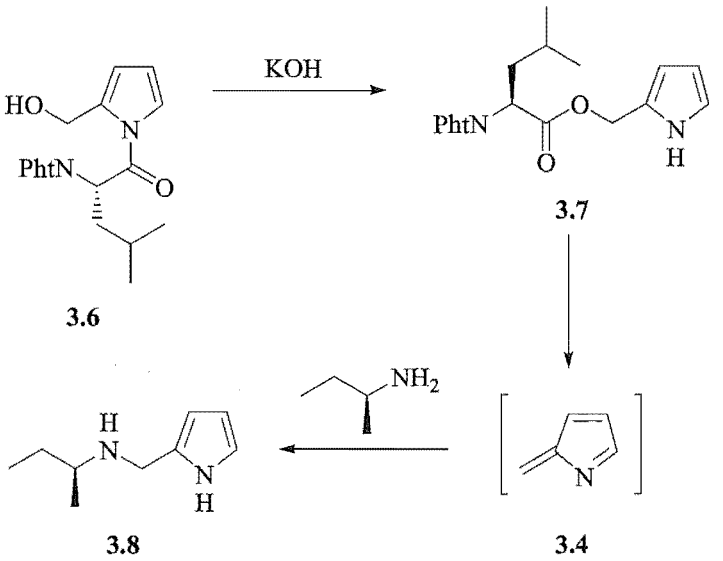
posed interaction with the active site of α-chymotrypsin is illustrated in Figure 3.1. The hydrophobic phenylsulfonyl EWG of **3.1a** was projected to mimic the aromatic P_1 group adjacent to the scissile peptide bond in natural substrates of α-chymotrypsin.[5c] (See Chapter One, Section 1.2 for a discussion of the S/P nomenclature developed by Schechter and Berger.[7]) The pyrrole ring was proposed to occupy the space where the P_1' residue normally resides in the enzyme active site. Hence, these peptidomimetics were postulated to be dipeptide analogues of an α-chymotrypsin substrate.

2-(Hydroxymethyl)pyrroles based on structure **3.1** were designed to act as mechanism-based inhibitors of α-chymotrypsin based on extensive chemical precedence. In particular, Scheme 3.1 outlines the possible pathways for nucleophilic attack upon the *N*-substituted 2-(hydroxymethyl)pyrrole **3.1** and its unsubstituted analogue **3.3**. The presence of an EWG, such as triflyl or mesyl, on **3.1** has been shown by deuterium labelling experiments to favour displacement of the hydroxyl group by an S_N2 mechanism to give **3.5a** (pathway b), rather than via the azafulvene intermediate **3.2** (pathway a).[8] Unsubstituted 2-(hydroxymethyl)pyrroles such as **3.3** readily formed azafulvene **3.4**, and subsequent nucleophilic attack resulted in compounds of type **3.5b**.[9]



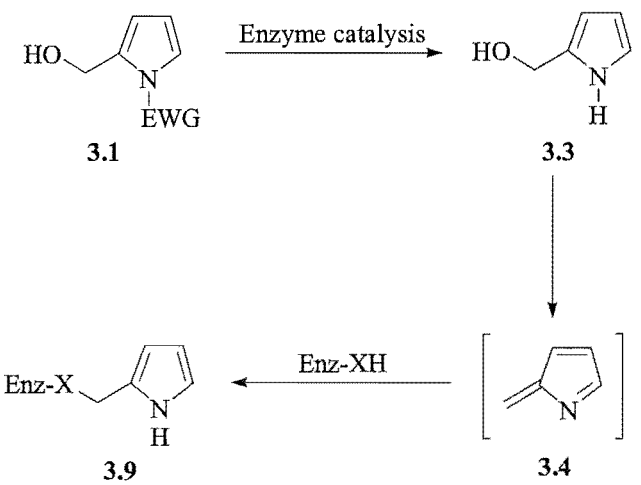
Scheme 3.1. Reaction of the 2-(hydroxymethyl)pyrroles **3.1** and **3.3** with nucleophiles to give **3.5a-b**.

Further analysis of *N*-substituted 2-(hydroxymethyl)pyrroles revealed that potassium hydroxide-catalysed transfer of the *N*-acyl EWG of **3.6** gave the 2-(*O*-acylhydroxymethyl)pyrrole **3.7**[10] (Scheme 3.2). The presence of an excellent leaving group allowed azafulvene formation to occur, and consequent nucleophilic attack by (+)-*sec*-butylamine afforded **3.8**. This system was proposed to model the mechanism-based inhibition of serine proteases, with the hydroxide mimicking the Ser195 residue and the external nucleophile mimicking the final step of covalent attachment to the enzyme.



Scheme 3.2. Reaction sequence determined for **3.6**, which ultimately gave **3.8**.

The experimental evidence presented here led to the development of a general mechanism of enzyme inhibition for 2-(hydroxymethyl)pyrroles of type **3.1**. Enzyme catalysed removal of the EWG from **3.1** releases the latent reactivity of this molecule (Scheme 3.3). Azafulvene **3.4** is then formed, and subsequent reaction with an active site nucleophile affords the enzyme-inhibitor adduct **3.9**.



Scheme 3.3. Mechanism-based inhibition of serine proteases by **3.1**.

A recent report from these laboratories determined that the mode of binding displayed by the representative 2-(hydroxymethyl)pyrrole **3.1a** to α-chymotrypsin was in fact non-covalent in nature.[11] Incubation of **3.1a** with α-chymotrypsin revealed signals in the LCMS spectrum at 25,236 g mol^{-1} (corresponding to native enzyme) and 25,473 g mol^{-1}. The difference of 237 g mol^{-1} corresponded to the molecular mass of intact **3.1a**. Incorporation of labelling at the hydroxymethyl carbon of **3.1a**, followed by incubation with α-chymotrypsin, gave a resonance of δ 56 ppm in the ^{13}C NMR spectrum, the same chemical shift observed for labelled **3.1a** in the absence of enzyme. The lack of covalent binding displayed by **3.1a** towards α-chymotrypsin stimulated an investigation towards the discovery of alternative pyrrole-based structures as potential mechanism-based α-chymotrypsin inhibitors. Introduction of an EWG at the C2 position for a series of pyrrole-based inhibitors was considered a means to promote covalent bond formation between the enzyme and inhibitor, as opposed to attachment of the EWG to the nitrogen as in compounds of type **3.1**. It was reasoned that the pyrrole ring system would be deactivated to a lesser extent, and as a result azafulvene formation and covalent derivatisation of the enzyme would be more likely to occur. Figure 3.2 illustrates the general structure of the proposed inhibitors (**3.10**), compared to that of the natural substrate, in addition to the in-

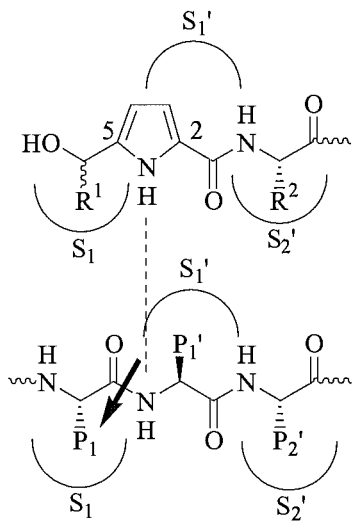
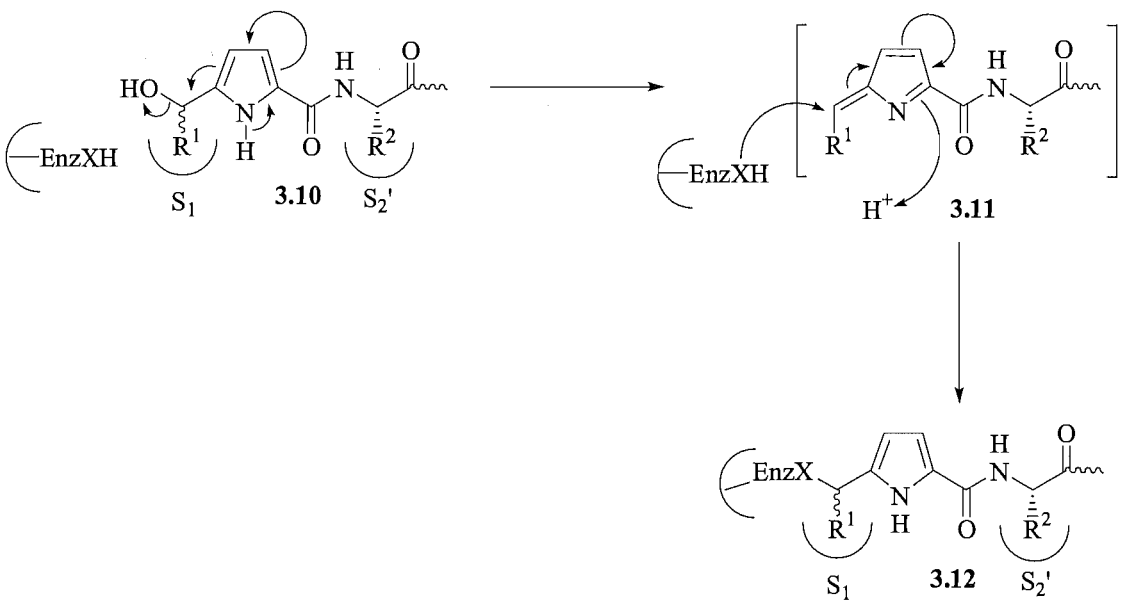


Figure 3.2. Schematic representation of the substrate (bottom) bound to the active site of α-chymotrypsin, correlated to the structure of the C2-acyl-C5-(hydroxyalkyl)pyrrole **3.10**. The point of cleavage of the natural substrate is indicated by a bold arrow.

teraction of both structures with the active site of α-chymotrypsin. An acyl group is attached to the C2 position, and its electron withdrawing properties are lessened by the occurrence of electron delocalisation in the amide bond. Note that **3.10** mimics a more favourable substrate conformation compared to that mimicked by **3.1a** in Figure 3.1.

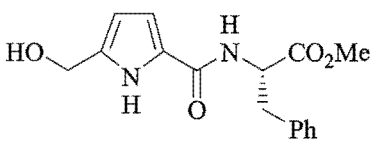
Covalent linkage of **3.10** to α-chymotrypsin would be favoured by formation of the azafulvene **3.11** (Scheme 3.4), which would then react with an active site residue to give the enzyme-inhibitor adduct **3.12**. The mechanistic sequence displayed here differs to that for **3.1** (see Scheme 3.3), as formation of the azafulvene **3.10** is not dependant on release of an EWG. Elaboration of **3.10** at positions R^1 and R^2 would allow compounds of this type to more closely mimic the natural substrate, which would potentially elicit a more potent inhibitory response to that of the *N*-substituted inhibitors. A series of such compounds was prepared and assayed against α-chymotrypsin. The solid phase synthesis of a representative C2-acyl-5-(hydroxymethyl)pyrrole-based α-chymotrypsin inhibitor was also investigated.



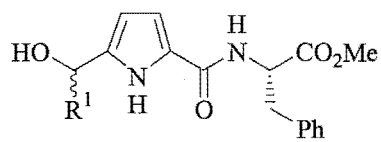
Scheme 3.4. Mechanism-based inhibition of serine proteases by the C2-acyl-C5-(hydroxyalkyl)pyrrole **3.10**.

3.2 PREPARATION AND ASSAY OF C2-ACYL-C5-(HYDROXYALKYL)-PYRROLES TO DETERMINE THE OPTIMAL GROUP AT R^1 FOR α-CHYMOTRYPSIN INHIBITION

The initial focus of this research was to understand the importance of R^1 in compounds of type **3.10** (see Figure 3.2) with respect to α-chymotrypsin inhibition. Three compounds were targeted for synthesis – the 5-(hydroxymethyl)pyrrole **3.13a** and the C5-(hydroxyalkyl)pyrroles **3.14a-b** (Figure 3.3). Compound **3.13a** has a hydroxymethyl group at the C5 position to allow for comparison of its inhibitory properties and those of the *N*-substituted 2-(hydroxymethyl)pyrroles synthesised in the previous study.[6] The 1-hydroxy-3-phenylpropyl side chain of **3.14a** was chosen to mimic the aromatic recognition element at P_1 of the natural substrate of α-chymotrypsin (see Figure 3.2). The 1-hydroxyethyl side chain of **3.14b** was chosen to be of intermediary size and hydrophobicity between those given by the small hydroxymethyl group of **3.13a** and the bulky aromatic group of **3.14a**. Determination of the inhibitory activity for **3.13a** and **3.14a-b** would give insight into the importance of side chain size and hydrophobicity at the C5 position. The phenylalanine derivative was chosen to be appended at the C2 position as in our proposed inhibitor-enzyme interaction the aromatic side chain would fit into the S_2' subsite of α-chymotrypsin, which can accommodate large, hydrophobic moieties.[12] The following section details the synthetic methodology utilised in the preparation of **3.13a** and **3.14a-b**, in addition to the assay results for all three compounds.



3.13a



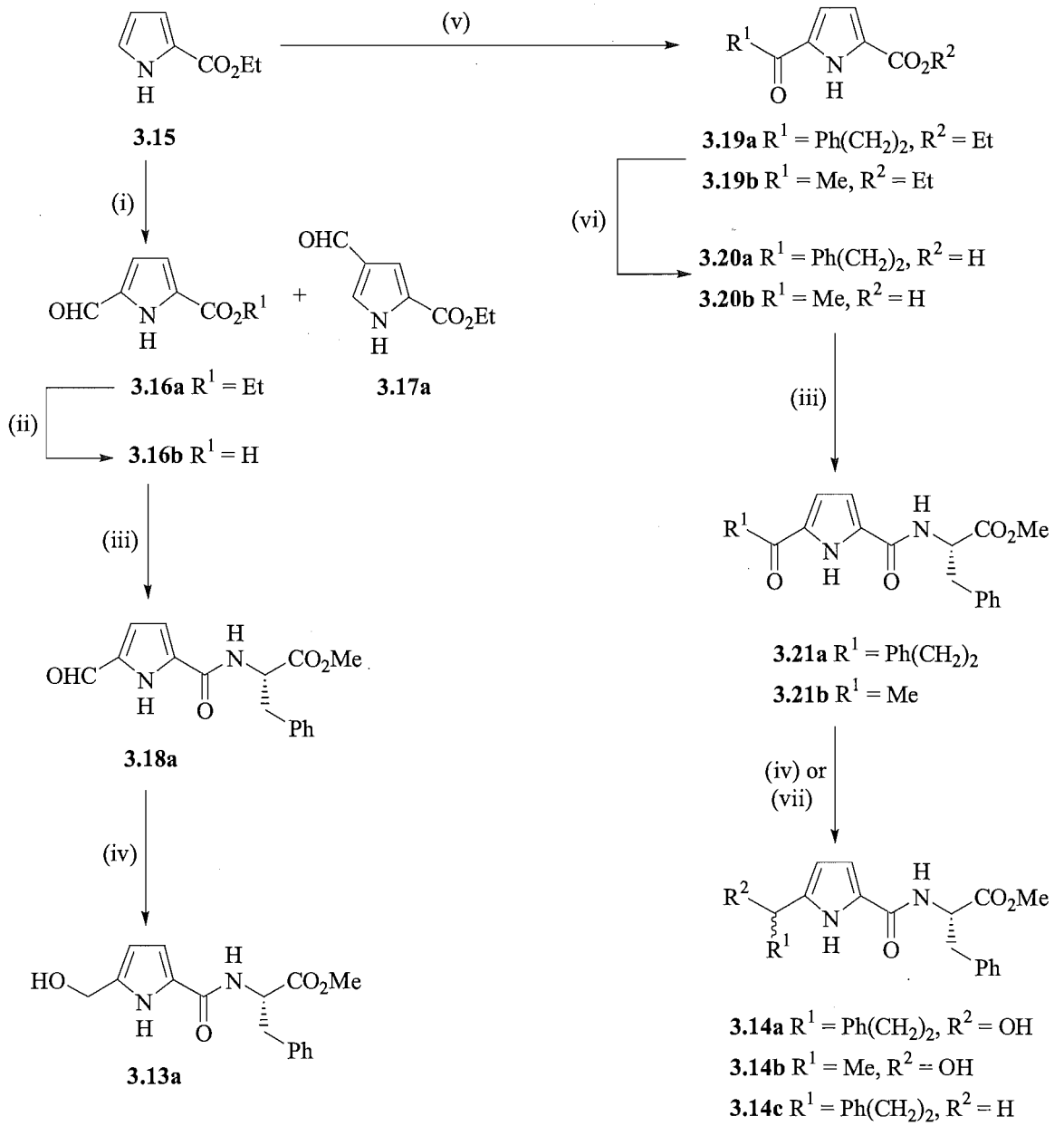
3.14a $R^1 = (CH_2)_2Ph$

3.14b $R^1 = Me$

Figure 3.3. Synthetic targets **3.13a** and **3.14a-b**.

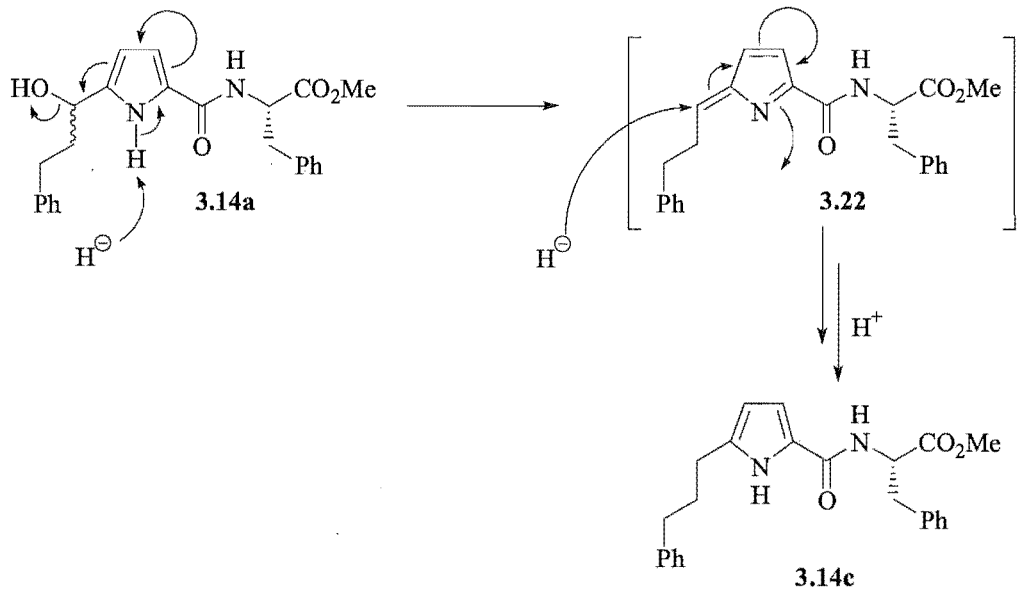
3.2.1 Preparation of peptidomimetics 3.13a and 3.14a-b

Scheme 3.5 details the synthesis of compounds **3.13a** and **3.14a-b**. The pyrrole ester **3.15**[13] was formylated under Vilismeier-Haack conditions[14] to give **3.16a** and its 4-formyl isomer **3.17a** in 61% and 25% yields respectively. Hydrolysis of the ester **3.16a**[15] with potassium hydroxide afforded the acid **3.16b** in a yield of 60%. Acid **3.16b** was coupled to L-phenylalanine methyl ester hydrochloride under standard EDCI/HOBT conditions[16] to give **3.18a** in 71% yield, and subsequent reduction of the 5-formylpyyrole **3.18a** with zinc borohydride[8] afforded the tripeptide analogue **3.13a** in a moderate yield of 57%. The C5-(hydroxyalkyl)pyrroles **3.14a-b** were obtained by an analogous synthetic route. Pyrrole ester **3.15** was acylated separately by hydrocinnamoyl chloride and acetyl chloride[17] to give **3.19a-b** in 35% and 37% yields respectively. The pyrrole esters **3.19a-b** were subjected to hydrolysis by potassium hydroxide to afford the acids **3.20a-b** in yields of 86% and 93% respectively. A 2:1 water/tetrahydrofuran solvent system was necessary for hydrolysis of **3.19a-b** due to the insufficient solubility of these starting materials in the 100% water system used for hydrolysis of **3.16a**. The acids **3.20a-b** were separately coupled to L-phenylalanine methyl ester hydrochloride under EDCI/HOBT conditions to afford **3.21a-b** in 70% and 91% yields respectively. Compound **3.21a** was reduced by zinc borohydride to give the extended peptide analogue **3.14a** in a relatively low yield of 21% as an inseparable 1:1 mixture of diastereoisomers by ^{1}H and ^{13}C NMR spectroscopy. Additionally, the over-reduced byproduct **3.14c** was obtained after flash chromatography in an approximately 1:1 mixture with unreacted **3.21a** by ^{1}H NMR spectroscopy. Reduction of **3.21b** with lithium borohydride afforded **3.14b** in 46% yield, but this compound proved difficult to purify and was ultimately unstable by ^{1}H NMR spectroscopy, as a complicated mixture of byproducts had formed.



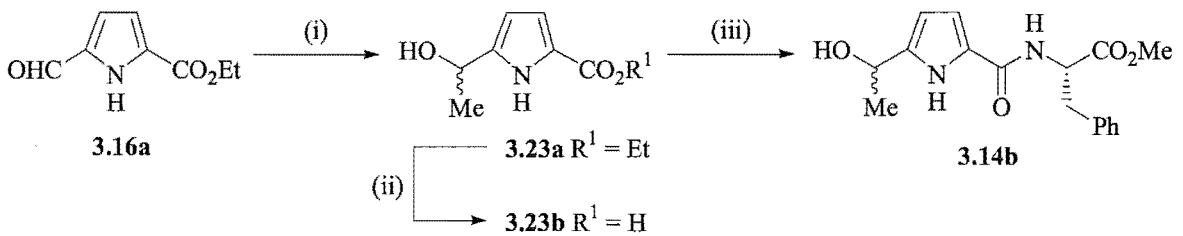
Scheme 3.5: *Reagents and conditions:* (i) $POCl_3$, DMF, 1,2-DCE, reflux, 15 min, then $CH_3CO_2Na.3H_2O$, reflux, 15 min (**3.16a**, 61%), (**3.17a**, 25%). (ii) KOH, H_2O, 40-50°C, 2 h (60%). (v) L-PheOMe.HCl, EDCI, HOBT, DIEA, CH_2Cl_2, r.t., 16 h (**3.18a**, 71%), (**3.21a**, 70%), (**3.21b**, 91%). (iv) $Zn(BH_4)_2$, Et_2O, 0°C, 1 h (**3.13a**, 57%), (**3.14a**, 21%), (**3.14c**). (v) $ZnCl_2$, 1,2-DCE, 50°C, 1 h and $Ph(CH_2)_2COCl$ (**3.19a**, 35%) or AcCl (**3.19b**, 37%). (vi) KOH, 2:1 H_2O/THF, 40-50°C, 2 h (**3.20a**, 86%), (**3.20b**, 93%). (vii) $LiBH_4$, Et_2O, -78°C, 1 h, then r.t., 1 h (**3.14b**, 46%).

The over-reduction of **3.14a** was proposed to occur via the mechanism outlined in Scheme 3.6. Hydride-induced formation of the azafulvene **3.22**, which subsequently reacted with a hydride nucleophile, gave **3.14c** upon workup.



Scheme 3.6. Proposed mechanism for the over-reduction of **3.14a** to give **3.14c**.

Compound **3.14b** was also prepared by the methodology presented in Scheme 3.7. To this end, the formyl group of pyrrole **3.16a** underwent reaction with methyllithium to give **3.23a** in 95% yield. Ethyl ester hydrolysis with sodium hydroxide gave the acid **3.23b** in a low yield of 17%, which was subsequently coupled to L-phenylalanine methyl ester under standard EDCI/HOBT conditions to afford **3.14b** in 54% yield. This product again proved

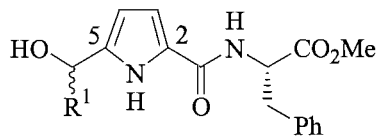


Scheme 3.7: *Reagents and conditions:* (i) MeLi, THF, -25°C, 5 h (95%). (ii) NaOH, 1:1 THF/H_2O, 40-50°C, 24 h (17%). (iii) L-PheOMe.HCl, EDCI, HOBT, DIEA, CH_2Cl_2, r.t., 16 h (54%).

to be unstable by the development of a complicated mixture, as determined by ^{1}H and ^{13}C NMR spectroscopy.

3.2.2 Assay results for peptidomimetics 3.13a and 3.14a

The inhibition results displayed in Table 3.1 for **3.13a** and **3.14a** against α-chymotrypsin were generated by a simple screening assay described previously.[6] (For a discussion of this assay technique, see Chapter Two, Section 2.3 – for the assays performed in this chapter, the $ZnCl_2$/EDTA solutions were replaced with distilled water.) Compound **3.14a** was assayed as the 1:1 mixture of diastereoisomers, and **3.14b** was too unstable to be assayed. Inhibition was measured at concentrations of 1250 μg mL^{-1}, 125 μg mL^{-1} and 12.5 μg mL^{-1}, made up separately for both **3.13a** and **3.14a.** The calculated percent inhibition values are a measure of the enzyme activity relative to that when no enzyme was present (no substrate turnover, 100% "inhibition") and when no inhibitor was present (full substrate turnover, 0% inhibition).



No.	R^1	% Inhibition (μg mL^{-1})		
		1250	125	12.5
3.13a	H	75	35	45
3.14a	$Ph(CH_2)_2$	75	35	15

Table 3.1. Inhibition of α-chymotrypsin by **3.13a** and **3.14a**.

No significant difference in inhibitor potency was observed between **3.13a** and **3.14a**, despite the presence of the bulky aromatic group present at R^1 of **3.14a** which was anticipated to occupy the primary specificity pocket S_1 (see Figure 3.2). The minimal

difference in activity observed between **3.13a** and **3.14a** suggested that these inhibitors do not interact with the enzyme active site in the manner proposed in Scheme 3.4. Inhibition of α-chymotrypsin can be elicited by a wide range of compounds, with the stipulation that an aromatic group is present.[18] It was decided from this outcome that the 5-(hydroxymethyl)pyrrole moiety was a sufficient scaffold upon which to focus further structure-activity studies, specifically on modifications at the C2 position. Additionally, determination of how **3.13a** binds to the active site of α-chymotrypsin would allow for comparison of its mode of inhibition to that of the *N*-substituted 5-(hydroxymethyl)pyrrole **3.1a**.

3.3 WORK TOWARDS DETERMINATION OF THE BINDING MODE DISPLAYED BY 3.13a TO α-CHYMOTRYPSIN

Mass spectrometry was performed on α-chymotrypsin, incubated with **3.13a** at 37°C for 3 hours in 50 mM Tris-HCl (pH 7.6), to determine whether or not the inhibitor was covalently attached to the enzyme. The mass spectrum obtained from incubation of the enzyme without **3.13a** is displayed in Figure 3.4. The four main signals could have resulted from how the enzyme responded to mass spectrometry, or the presence of α-chymotrypsin isoforms, or partially cleaved forms of the native enzyme. At least one of these forms was active, as the inhibitor assay demonstrated that hydrolysis of the substrate occurred, which made it a suitable enzyme sample for the mass spectrometry study performed here.

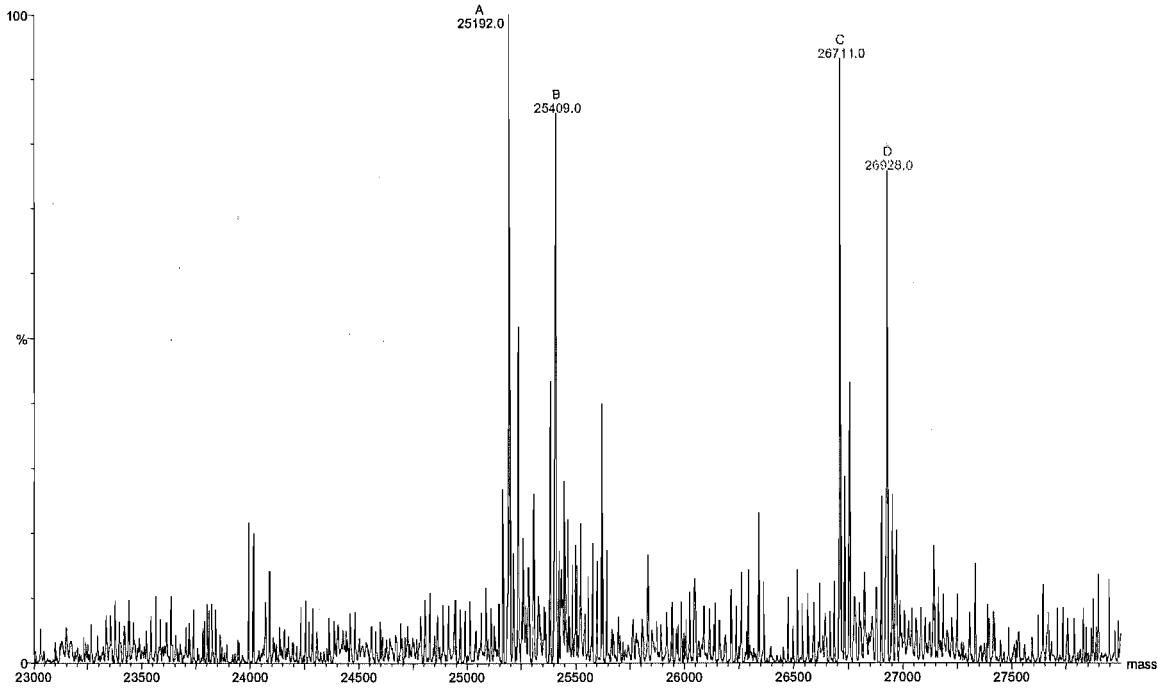


Figure 3.4. Mass spectrum of α-chymotrypsin incubated in Tris-HCl at 37°C for 3 h.

A separate incubation was performed under the same conditions outlined before, except for the addition of one equivalent of inhibitor **3.13a**. Figure 3.5 illustrates that an essentially identical spectrum was obtained to that given by the enzyme alone (Figure 3.4). The only noticeable differences are slight variations in the peak heights of the four major signals, *e.g.* in Figure 3.4 peak A was the base peak, whereas in Figure 3.5 peak B was the signal of greatest intensity. No increase in mass of 285.3 g mol^{-1} was observed for any of the four main signals, which was the increase expected if **3.13a** was covalently bound to an active site nucleophile as a result of the azafulvene-type mechanism proposed in Scheme 3.4. Therefore, inhibitor **3.13a** must act as a non-covalent, reversible inhibitor in a manner similar to that determined for **3.1a** (see Introduction to this chapter).

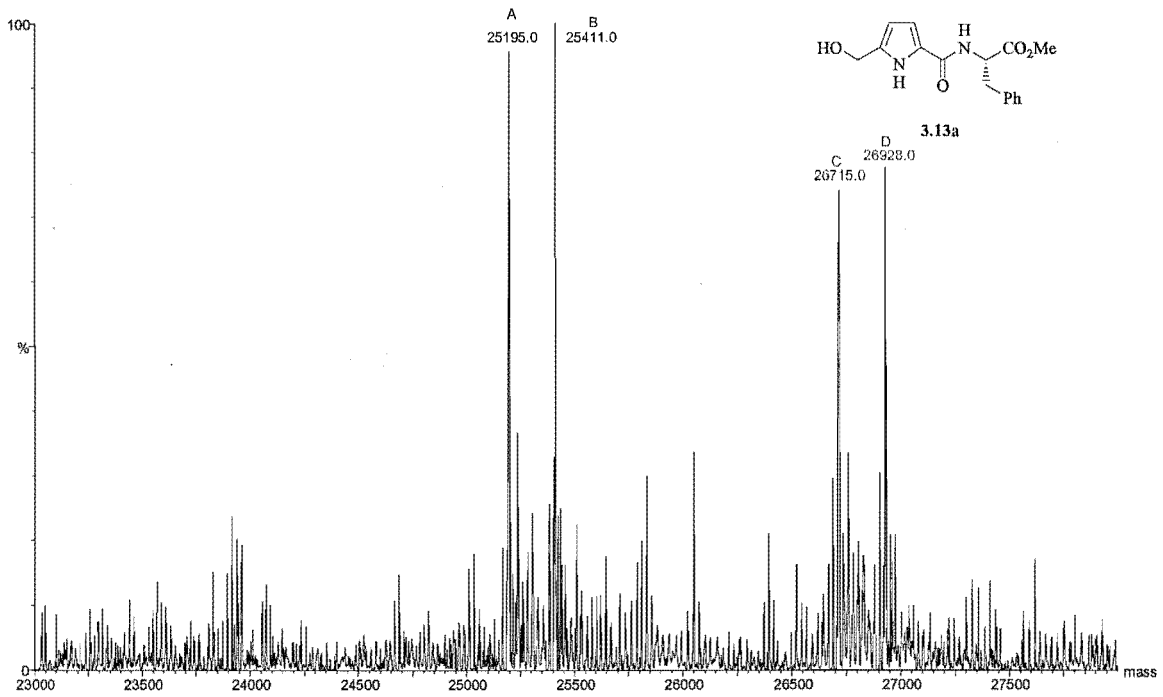


Figure 3.5. Mass spectrum of α-chymotrypsin and **3.13a** incubated in Tris-HCl at 37°C for 3 h.

3.4 PREPARATION AND ASSAY OF C2-ACYL-5-(HYDROXYMETHYL)-PYRROLES TO DETERMINE THE OPTIMAL GROUP AT R^2 FOR α-CHYMOTRYPSIN INHIBITION

In light of the finding that **3.13a** did not appear to be covalently bound to α-chymotrypsin, further work was undertaken to increase the range of peptidomimetics of type **3.13a** for assay against α-chymotrypsin. This would result in a greater understanding of the structure-activity relationships for C2-acyl-5-(hydroxymethyl)pyrroles as reversible α-chymotrypsin inhibitors. All structural modifications in this section of work were restricted to the amino acid group at the C2 position. Replacement of the aromatic phenylalanine residue of **3.13a** with leucine and proline allowed evaluation of the inhibition elicited by an aliphatic (**3.13b**, Figure 3.6) or a cyclic, non-aromatic side chain (**3.24**), compared to that of **3.13a**. Additionally, an inhibitor was synthesised with a

dipeptide functionality at the C2 position (**3.25**). The L-phenylalanyl-L-leucine dipeptide portion of **3.25** was chosen as previous studies[19] have shown that a leucine residue contiguous to a phenylalanine elicited greater inhibition of α-chymotrypsin, in comparison to inhibitors that only possessed a phenylalanine residue.

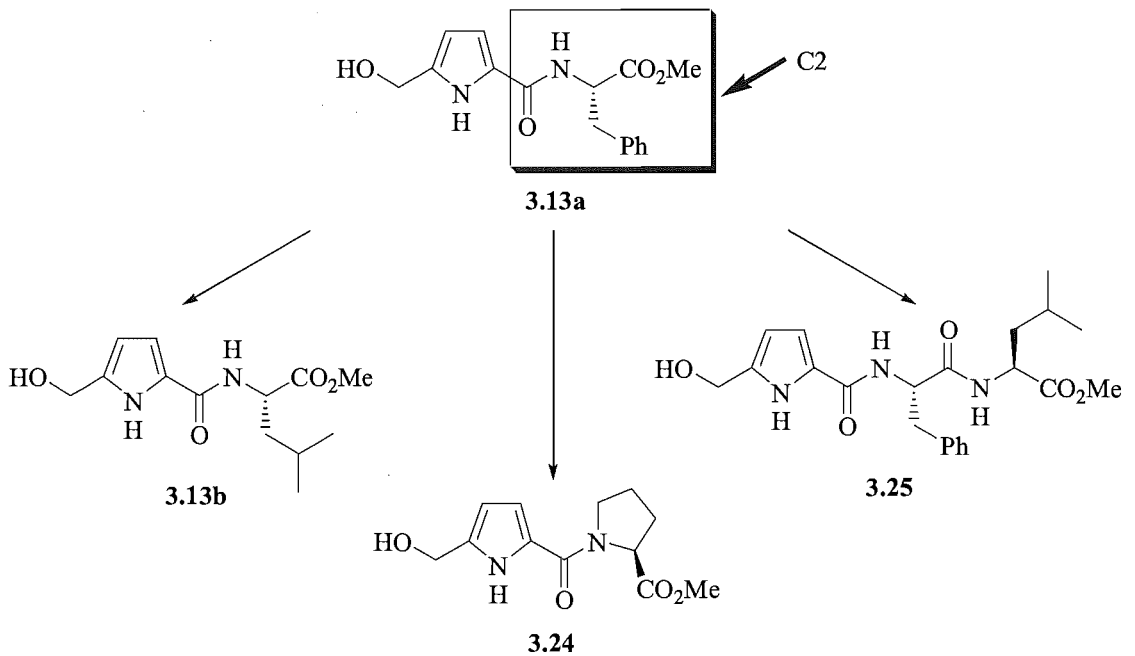
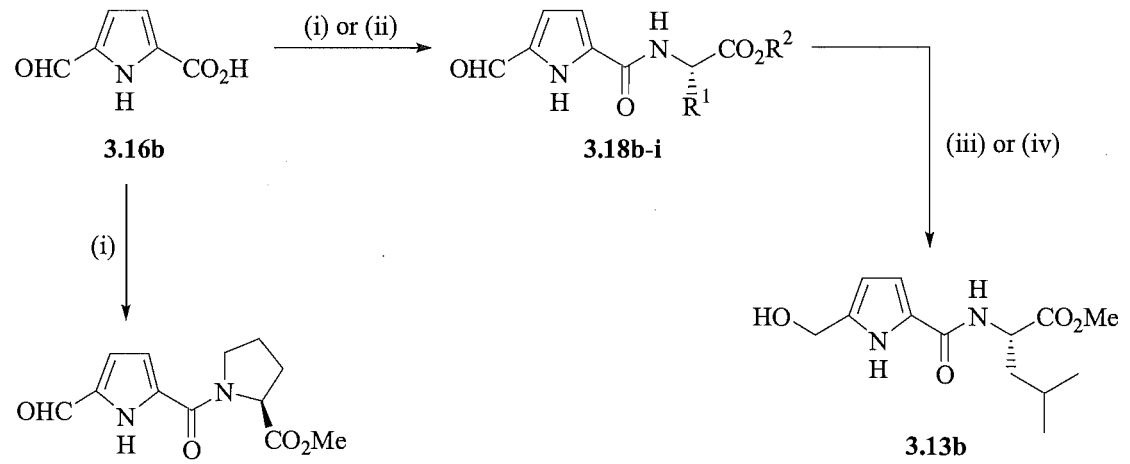


Figure 3.6. Structural modifications to the α-chymotrypsin inhibitor **3.13a** at C2 afforded the synthetic targets **3.13b**, **3.24** and **3.25**

3.4.1 Preparation of peptidomimetics 3.13b, 3.24 and 3.25

Preparation of **3.13b** and **3.24** was performed as described for the synthesis of **3.13a**. To this end, **3.16b** was coupled under standard EDCI/HOBT conditions to L-leucine methyl ester hydrochloride and L-proline methyl ester hydrochloride to give **3.18b** and **3.26** in yields of 61% and 74% respectively (Scheme 3.8). Separate reduction of **3.20b** and **3.26** with zinc borohydride afforded the tripeptide mimics **3.13b** and **3.24** in 42% and 24% yields respectively. The yield of **3.13b** was improved to 66% with the use of lithium borohydride as the reducing agent in place of zinc borohydride. Scheme 3.8 also presents the separate EDCI/HOBT-mediated couplings between **3.16b** and glycine ethyl ester

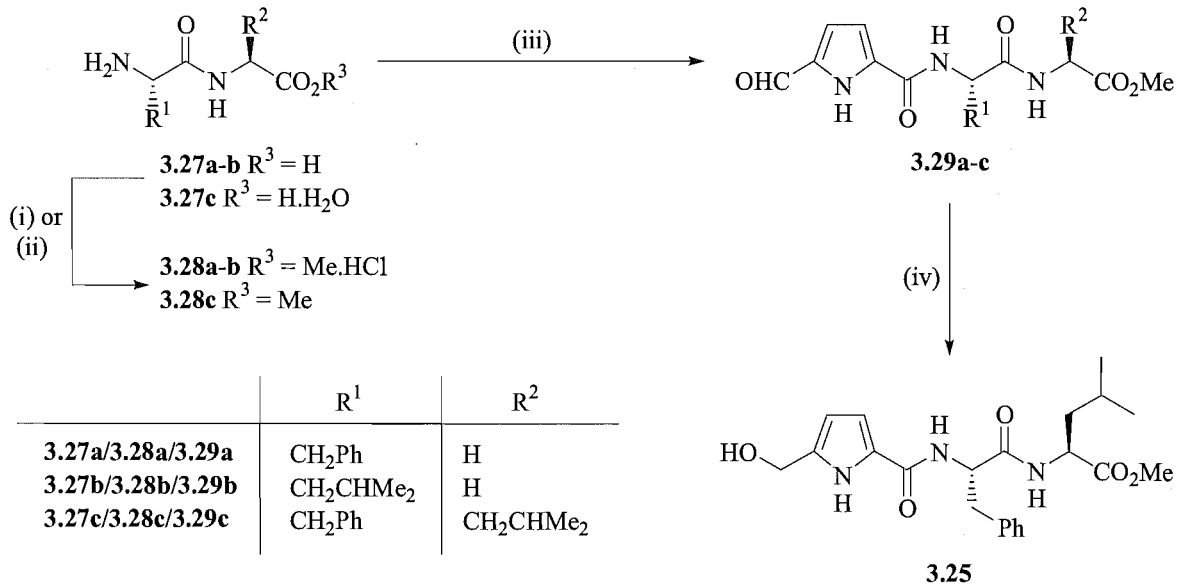
hydrochloride,[20] glycine benzyl ester hydrochloride, L-phenylalanine ethyl ester hydrochloride, L-leucine ethyl ester hydrochloride, L-valine methyl ester hydrochloride and L-alanine methyl ester hydrochloride to give **3.18c-h** in yields of 70%, 56%, 58%, 38%, 53% and 43% respectively. Acid **3.16b** and glycine methyl ester hydrochloride were coupled using BOP[21] to afford **3.18i** in 44% yield, as this amino acid derivative did not couple to **3.16b** under standard EDCI/HOBT conditions. Intermediates **3.18a-i** were used in the development of a novel *N*-derivatising agent for amino acids (see Chapter Four for further discussion).



	R^1	R^2
3.18b	CH_2CHMe_2	Me
3.18c (ref 20)	H	Et
3.18d	H	Bn
3.18e	CH_2Ph	Et
3.18f	CH_2CHMe_2	Et
3.18g	$CHMe_2$	Me
3.18h	Me	Me
3.18i	H	Me

Scheme 3.8. *Reagents and conditions:* (i) EDCI, HOBT, DIEA, CH_2Cl_2, r.t., 16 h and either L-LeuOMe.HCl (**3.18b**, 61%), GlyOEt.HCl (**3.18c**, 70%),[20] GlyOBn.HCl (**3.18d**, 56%), L-PheOEt.HCl (**3.18e**, 58%), L-LeuOEt.HCl (**3.18f**, 38%), L-ValOMe.HCl (**3.18g**, 53%) or L-AlaOMe.HCl (**3.18h**, 43%) or L-ProOMe.HCl (**3.26**, 74%). (ii) GlyOMe.HCl, BOP, DIEA, CH_2Cl_2, r.t., 16 h (**3.18i**, 44%). (iii) $Zn(BH_4)_2$, ether, 0°C, 1 h (**3.13b**, 42%), (**3.24**, 24%). (iv) $LiBH_4$, ether, -78°C, 1 h (**3.13b**, 66%).

Preparation of the tetrapeptide analogue **3.25** began with the esterification of L-phenylalanyl-L-leucine hydrate (**3.27c**, Scheme 3.9) by diazomethane, after which the resultant methyl ester **3.28c** was coupled to **3.16b** in the presence of BOP to afford **3.29c** in 52% yield over two steps. Reduction with lithium borohydride then gave **3.25** in 68% yield. The dipeptide-pyrrole adducts **3.29a-b** were synthesised by an analogous route to that of **3.29c** for use in Chapter Four. To this end, the dipeptides L-phenylalanylglycine **3.27a** and L-leucylglycine **3.27b** (Scheme 3.9) were esterified with oxalyl chloride/catalytic DMF in refluxing methanol to give the methyl ester hydrochlorides **3.28a-b** in 89% and quantitative yields respectively. Separate reaction of **3.28a-b** with **3.16b** via BOP-mediated couplings gave **3.29a-b** in yields of 49% and 45% respectively.

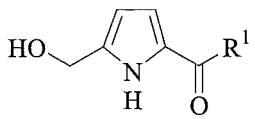


	R^1	R^2
3.27a/3.28a/3.29a	CH_2Ph	H
3.27b/3.28b/3.29b	CH_2CHMe_2	H
3.27c/3.28c/3.29c	CH_2Ph	CH_2CHMe_2

Scheme 3.9: *Reagents and conditions:* (i) $(COCl)_2$, $DMF_{(cat.)}$, MeOH, reflux, 3 h (**3.28a**, 89%), (**3.28b**, quantitative yield). (ii) CH_2N_2, THF, r.t., 15 h (**3.28c**). (iii) **3.16b**, BOP, DIEA, CH_2Cl_2, r.t., 16 h (**3.29a**, 49%), (**3.29b**, 45%), (**3.29c**, 52% over two steps). (iv) $LiBH_4$, THF, -78°C, 1 h, then r.t., 1 h (68%).

3.4.2 Assay results for peptidomimetics 3.13b, 3.24 and 3.25

Assay results for compounds **3.13b**, **3.24** and **3.25** against α-chymotrypsin are displayed in Table 3.2. The assay conditions used to determine the inhibitory activity of **3.13b**, **3.24** and **3.25** were identical to that used for **3.13a** and **3.14a**. The results for **3.13a** (which initially appeared in Table 3.1) are included for comparison.



No.	R^1	% Inhibition (μg mL^{-1})		
		1250	125	12.5
3.13a	NH-L-PheOMe	75	35	45
3.13b	NH-L-LeuOMe	10	5	20
3.24	N-L-ProOMe	45	25	5
3.25[¶]	NH-L-Phe-L-LeuOMe	100	20	15

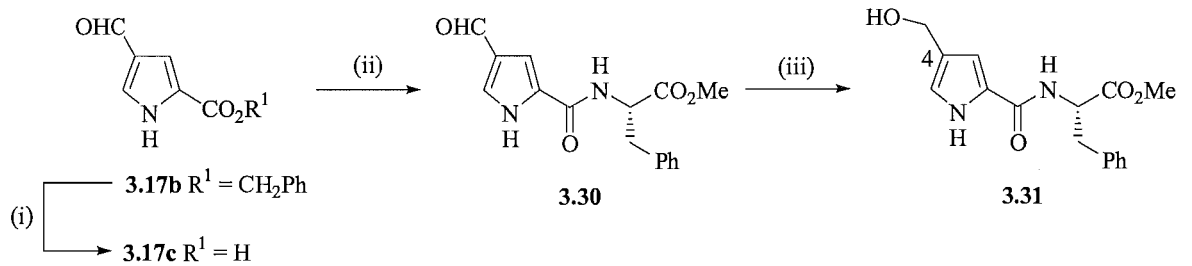
[¶] IC_{50} = 108 μM.

Table 3.2. Inhibition of α-chymotrypsin by **3.13a-b**, **3.24** and **3.25**.

Comparison of the assay results for compounds that possessed a 5-(hydroxymethyl)pyrrole moiety (**3.13a-b**, **3.24** and **3.25**) demonstrated that those with a phenylalanine residue at the C2 position (**3.13a** and **3.25**) were the most potent. The increased activity of **3.13a** and **3.25** was consistent with α-chymotrypsins preference for aromatic-containing substrates and inhibitors. A proline residue (as in **3.24**) gave increased potency relative to the acyclic aliphatic leucine residue in **3.13b**. However, both compounds were noticeably less active than **3.13a** and **3.25**. Inhibitor **3.25** gave the greatest activity against α-chymotrypsin of the compounds assayed, and a more in-depth study led to the determination of an IC_{50} value of 108 μM. This result revealed that **3.25** was an effective inhibitor of α-chymotrypsin.

3.5 ATTEMPTED SYNTHESIS OF AN α-CHYMOTRYPSIN INHIBITOR WITH A 4-(HYDROXYMETHYL)PYRROLE FUNCTIONALITY

An investigation of alternative pyrrole-based scaffolds to that of **3.13a-b**, **3.14a**, **3.24** and **3.25** was undertaken to determine whether greater inhibition could be elicited, in addition to a covalent binding mode for the inhibitor to α-chymotrypsin. Synthesis of an alternative 4-(hydroxymethyl)pyrrole scaffold was targeted to elucidate whether transfer of the hydroxymethyl group from the C5 position to C4 would give the desired effects. To this end, the 4-formyl pyrrole ester **3.17b**[22] was hydrolysed with potassium hydroxide to give the acid **3.17c** in 77% yield (Scheme 3.10), which was subsequently coupled to L-phenylalanine methyl ester under standard EDCI/HOBT conditions to afford **3.30** in 66% yield. Reduction with lithium borohydride gave the 4-(hydroxymethyl)pyrrole **3.31** in a low yield of 28%. However, this compound degraded to a complicated mixture of byproducts within 1-2 days as demonstrated by ^{1}H NMR spectroscopy, before measurement of its activity as an α-chymotrypsin inhibitor could be performed. Further work towards the synthesis of 4-(hydroxymethyl)pyrrole-based α-chymotrypsin inhibitors was abandoned at this point.



Scheme 3.10. Reagents and conditions: (i) KOH, H_2O, 40-50°C, 2 h (77%). (ii) L-PheOMe.HCl, EDCI, HOBT, DIEA, CH_2Cl_2, r.t., 16 h (66%). (iii) $LiBH_4$, Et_2O, -78°C, 1 h, then r.t., 1 h (28%).

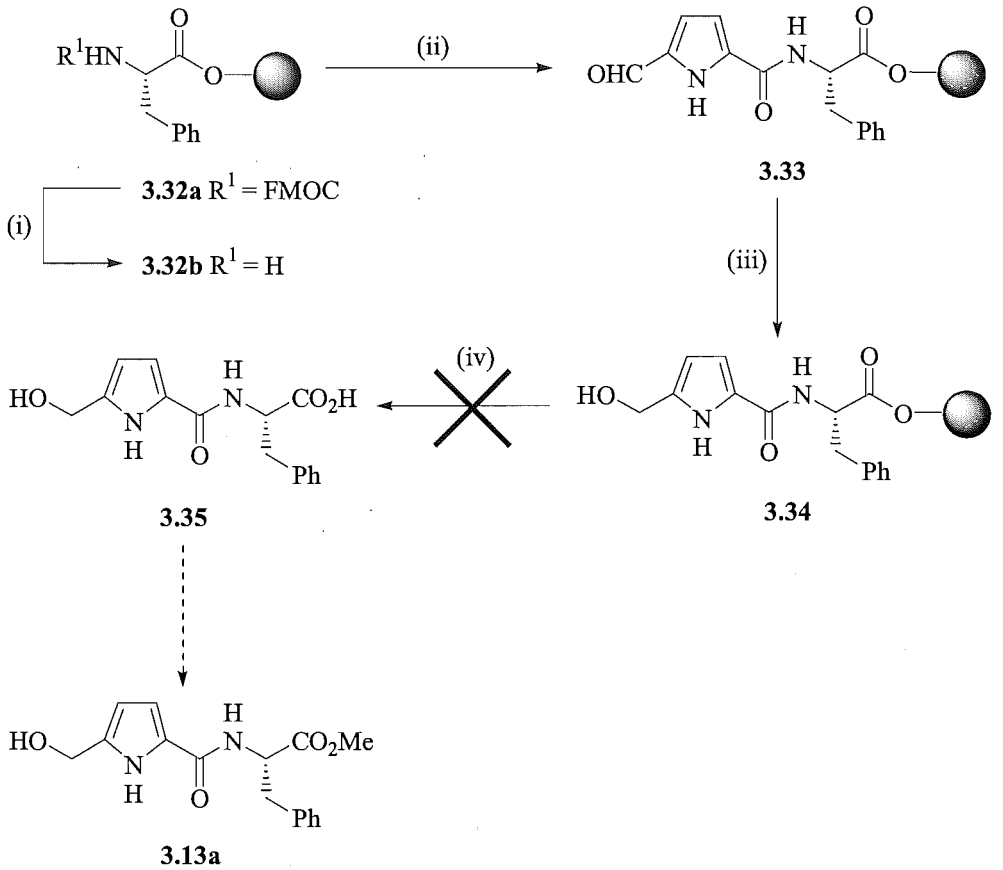
3.6 ATTEMPTED SOLID PHASE SYNTHESIS OF 5-(HYDROXYMETHYL)-PYRROLE-BASED α-CHYMOTRYPSIN INHIBITORS

The solid phase synthesis of an α-chymotrypsin inhibitor series with a 5-(hydroxymethyl)pyrrole moiety would allow for a broader examination of structure-activity relationships at the C2 position. Combinatorial synthesis is a powerful method for screening large collections of compounds against a biological target.[23] Synthesis of **3.13a** by solid phase chemistry was investigated as a model system for the development of a diversity-orientated synthetic route for this class of compounds.

3.6.1 Attempted reduction of 3.33, a solid phase precursor to 3.13a

The initial attempt towards development of a solid-phase based synthetic route to **3.13a** was based on one critical step. Reduction of a 5-formylpyrrole moiety, linked via its C2-acyl group to a resin-bound amino acid, would afford the desired 5-(hydroxymethyl)pyrrole functionality. This reduction step is analogous to that illustrated in Scheme 3.5, whereby **3.18a** was reacted with zinc borohydride to give **3.13a**. To this end, deprotection of *N*-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl Wang resin (**3.32a**, Scheme 3.11) with 1:4 piperidine/dichloromethane gave the free amine **3.32b**, and reaction with the pyrrole acid **3.16b** under BOP-mediated coupling conditions afforded **3.33**. Reaction of **3.33** with sodium borohydride in DMF[24] was performed in an effort to obtain the proposed resin-bound 5-(hydroxymethyl)pyrrole **3.34**, after which the free acid analogue of **3.13a** (**3.35**) would be released by cleavage from the resin with 1:1 TFA/dichloromethane. The ^{1}H NMR spectrum of the cleaved material indicated that extensive degradation had occurred and the expected product **3.35** was not present. Exposure of the 5-(hydroxymethyl)pyrrole **3.13a**, obtained by the solution phase synthetic route outlined in Scheme 3.5, to 1:1 TFA/dichloromethane at r.t. for 1 h resulted in extensive degradation of **3.13a** as determined by ^{1}H NMR spectroscopy. Conversely, the same conditions did not cause degradation of **3.18a**, the solution phase precursor to **3.13a,** which conversely possessed a 5-formylpyrrole moiety. These results indicated that the 5-

(hydroxymethyl)pyrrole functionality was not compatible with a cleavage procedure that involved the use of a strong acid. An alternative synthetic strategy needed to be developed that avoided formation of the 5-(hydroxymethyl)pyrrole moiety on the solid phase.

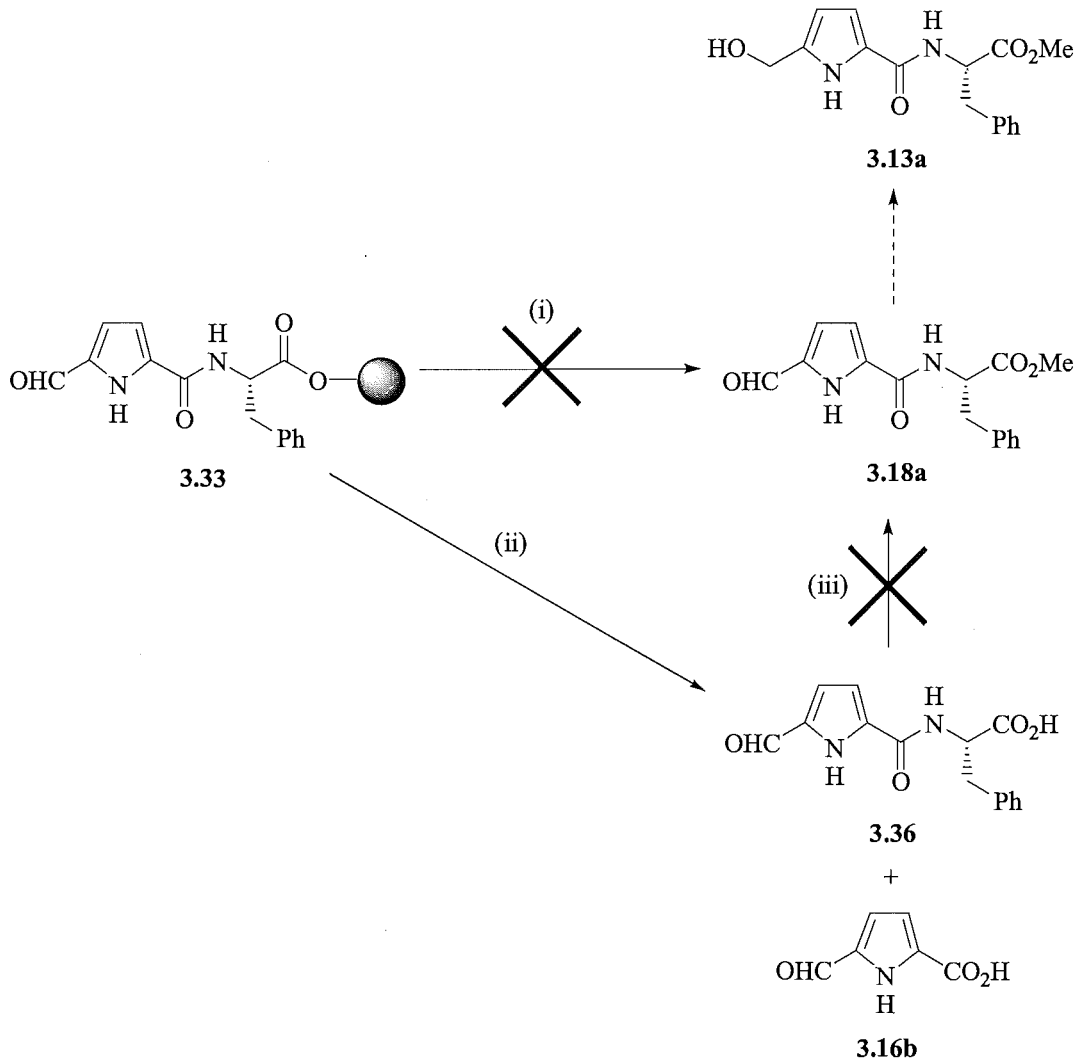


Scheme 3.11. *Reagents and conditions:* (i) 1:4 piperidine/dichloromethane, r.t., 30 min. (ii) **3.16b**, BOP, DIEA, CH_2Cl_2, r.t., 48 h. (iii) $NaBH_4$, DMF, r.t., 24 h. (iv) 1:1 TFA/CH_2Cl_2, r.t., 2 h.

3.6.2 Attempted preparation of 3.18a from its solid phase synthesis precursor 3.33

Scheme 3.12 outlines the attempted preparation of **3.18a**, the immediate precursor to the representative 5-(hydroxymethyl)pyrrole α-chymotrypsin inhibitor **3.13a** (see Scheme 3.5). Preparation of **3.18a** was considered a model system for development of a synthetic route whereby the 5-formylpyrrole precursors would be synthesised on the solid phase, with the desired 5-(hydroxymethyl)pyrroles afforded by subsequent solution phase

reduction. Formation of **3.18a** required the cleavage of **3.33** and subsequent esterification of the carboxylic acid. Cleavage and esterification of a solid phase-linked carboxylic acid to afford the corresponding methyl ester has been combined into a "one-pot" methodology by Scheeren *et al.*[25] Application of the reported conditions to **3.33**, where the resin was suspended in refluxing 30:6:1 benzene/methanol/triethylamine in the presence of catalytic potassium cyanide, gave a mixture of compounds by ^{1}H NMR spectroscopy, with no indication of the desired intermediate **3.18a**. Direct cleavage of **3.33** was effected with 1:1 trifluoroacetic acid/dichloromethane to give the free acid **3.36**. However, **3.36** was obtain-

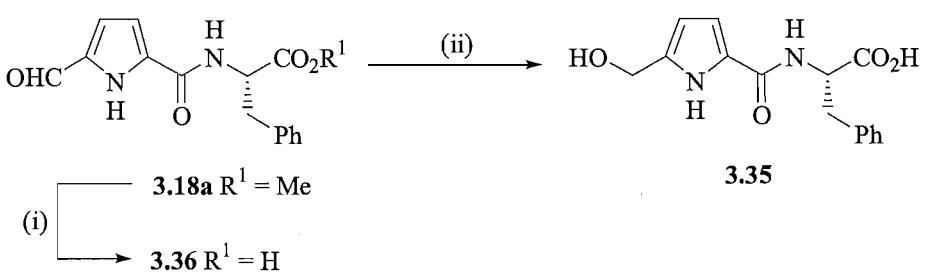


Scheme 3.12. *Reagents and conditions:* (i) 30:6:1 C_6H_6/MeOH/Et_3N, $KCN_{(cat.)}$, reflux, 48 h. (ii) 1:1 TFA/CH_2Cl_2, r.t., 2 h. (iii) CH_2N_2, THF, r.t., 15 h.

ed in an inseparable 1:1 mixture with unreacted **3.16b**, despite extensive washing of the resin after the coupling step that resulted in **3.33** (see Scheme 3.11). Improved isolation of the desired compound was attempted by the esterification of **3.36** with diazomethane to afford **3.18a**. Separation of **3.18a** from the methyl ester equivalent of **3.16b** was proposed to occur more readily than the separation of the acids **3.36** and **3.16b**, due to esters possessing more favourable solubility properties than acids for separation by flash chromatography. However, ^{1}H NMR analysis indicated that the pyrrole ring nitrogen was methylated by the electrophile under these conditions. This is in agreement with a previous report that noted the facile methylation of the pyrrole ring nitrogen with diazomethane.[26] Further work towards the solid phase-based synthesis of **3.13a** was abandoned at this point.

3.6.3 Attempted solution phase synthesis of peptidomimetic 3.35

Synthesis of the free acid **3.35** was also attempted by solution phase chemistry to determine whether it was a more potent inhibitor of α-chymotrypsin than its methyl ester derivative **3.13a**. To this end, **3.18a** was hydrolysed with potassium carbonate in refluxing 10:1 methanol/water[27] to give **3.36** in 82% yield (Scheme 3.13). Reduction of **3.36** with lithium borohydride in THF proved problematic, as the portionwise addition of 15 equivalents of the reducing agent resulted in only a 1:1 ratio of starting material to **3.35** by ^{1}H NMR spectroscopy. Reaction of **3.36** with sodium borohydride in 6:1 *iso*-propanol/water at r.t. afforded **3.35** in 44% yield. However, **3.35** was determined to be un-



Scheme 3.13. *Reagents and conditions:* (i) K_2CO_3, 10:1 MeOH/H_2O, reflux, 18 h (82%). (ii) $NaBH_4$, 6:1 *i*-PrOH/H_2O, r.t., 18 h (44%).

stable due to the gradual development of a complicated mixture of byproducts, as observed by ^{1}H NMR spectroscopy and electrospray LRMS.

3.7 CONCLUSION AND FUTURE WORK

A range of peptidomimetic mechanism-based inhibitors of α-chymotrypsin (**3.13a-b**, **3.14a**, **3.24** and **3.25**) was prepared and tested for inhibitory activity towards the target enzyme. These pyrrole-based compounds are distinguished by the presence of hydroxymethyl or hydroxyalkyl functionalities at the C5 position, in addition to a C2 acyl group. Irreversible inhibition was proposed to occur by formation of a reactive azafulvene upon entry into the enzymes active site, with subsequent formation of a covalent bond between the inhibitor and enzyme arising from nucleophilic attack by an active site residue. Preliminary results illustrated that the hydroxymethyl moiety at the C5 position of **3.13a** was sufficient for eliciting inhibition. Increased hydrophobicity and steric bulk at C5, in the form of the 3-phenyl-1-hydroxypropyl functionality present on **3.14a**, did not increase inhibitory activity. Analysis by mass spectrometry indicated that **3.13a** was not covalently attached to α-chymotrypsin, which suggested that this compound was in fact a reversible inhibitor. The importance of the aromatic group present at C2 of **3.13a/3.14a** for eliciting inhibition was illustrated by the lowered activity of compounds **3.13b** and **3.24**, which instead had non-aromatic leucine and proline residues at C2 respectively. Optimal inhibition of α-chymotrypsin was elicited by **3.25**, which had a phenylalanyl-leucine dipeptide at C2, and an IC_{50} of 108 μM was determined for this compound. Synthesis of compounds that possessed either a 5-(1-hydroxyethyl)pyrrole group (**3.14b**) or a 4-(hydroxymethyl)pyrrole functionality (**3.31**) gave unstable materials that could not be tested for inhibitory activity against α-chymotrypsin.

The attempted solid phase synthesis of **3.13a** demonstrated that the 5-(hydroxymethyl)pyrrole functionality was unstable to the acidic cleavage conditions used in the attempted release of **3.35**, a precursor to **3.13a**, from the solid support. Cleavage of

the resin-bound 5-formyl intermediate **3.33** gave the free acid **3.36** as a 1:1 mixture with unreacted **3.16b**, and attempts to purify this mixture were unsuccessful. The solution phase-based synthesis of **3.35** (the free acid analogue of **3.13a**), demonstrated that a compound of its structure, which possessed both hydroxyl and carboxylic acid functionalities, was unstable.

Future work could involve further elaboration of the functionality at the C2 position to determine if greater inhibition could be elicited. Additionally, esterification of the hydroxymethyl group at C5 could give more peptide-like structures and further enhancement of inhibition.

3.8 REFERENCES FOR CHAPTER THREE

1. Stroud, R. M. *Sci. Amer.* **1974**, *231*, 74.

2. Pochet, L.; Doucet, C.; Dive, G.; Wouters, J.; Masareel, B.; Reboud-Ravaux, M.; Pirotte, B. *Bioorg. Med. Chem.* **2000**, *8*, 1489.

3. (a) Janoff, A. *Ann. Rev. Med.* **1985**, *36*, 207. (b) Janoff, A. *Am. Rev. Respir. Dis.* **1985**, *132*, 417. (c) Snider, G. L. *Drug Dev. Res.* **1987**, *10*, 235. (d) Nadel, J. A. *Am. Rev. Respir. Dis.* **1991**, *14*, S48. (e) Demling, R. H. *Annu. Rev. Med.* **1995**, *46*, 193.

4. (a) Henderson, R.; Wright, C. S.; Hess, G. P.; Blow, D. M. *Cold Spring Harbor Symposia on Quantitative Biology* **1971**, *36*, 63. (b) Sigler, P. B.; Blow, D. M.; Matthews, B. W.; Henderson, R. *J. Mol. Biol.* **1968**, *35*, 143. (c) Matthews, B. W.; Sigler, P. B.; Henderson, R.; Blow, D. M. *Nature* **1967**, *214*, 652.

5. (a) Hedstrom, L. *Chem. Rev.* **2002**, *102*, 4501. (b) Perona, J. J.; Craik, C. S. *Prot. Sci.* **1995**, *4*, 337. (c) Kashima, A.; Inoue, Y.; Sugio, S.; Maeda, I.; Nose, T.; Shimohigashi, Y. *Eur. J. Biochem.* **1998**, *255*, 12.

6. Abell, A. D.; Nabbs, B. K. *Bioorg. Med. Chem.* **2001**, *9*, 621.

7. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.

8. Abell, A. D.; Nabbs, B. K.; Battersby, A. R. *J. Am. Chem. Soc.* **1998**, *120*, 1741.

9. (a) Barcock, R. A.; Moorcroft, N. A.; Storr, R. C.; Young, J. H.; Fuller, L. S. *Tetrahedron Lett.* **1993**, *34*, 1187. (b) Jones, R. A.; Bean, G. P. In *The Chemistry of Pyrroles*; Academic Press: London, 1977, pp. 425-438.

10. Abell, A. D.; Litten, J. C. *J. Chem. Soc., Chem. Commun.* **1998**, 919.

11. Martyn, D. C.; Vernall, A. J.; Clark, B. M.; Abell, A. D. *J. Chem. Soc., Org. Biomol. Chem.* **2003**, in press.

12. (a) Laskowski, J., M.; Tashiro, M.; Empie, M. W.; Park, S. J.; Kato, I.; Ardelt, W.; Wieczorek, M. In *Proteinase Inhibitors: Medical and Biological Aspects*; Katunuma, N., Umezawa, H., Holzer, H., Eds.; Springer-Verlag: Tokyo, 1983, pp. 55-68. (b) Imperiali, B.; Abeles, R. H. *Biochemistry* **1987**, *26*, 4474.

13. Bailey, D. M.; Johnson, R. E.; Albertson, N. F. *Org. Synth.* **1971**, *51*, 100.

14. Wallace, D. M.; Leung, S. H.; Senge, M. O.; Smith, K. M. *J. Org. Chem.* **1993**, *58*, 7245.

15. Khan, M. K. A.; Morgan, K. J.; Morrey, D. P. *Tetrahedron* **1966**, *22*, 2095.

16. Tian, Z.-Q.; Brown, B. B.; Mack, D. P.; Hutton, C. A.; Bartlett, P. A. *J. Org. Chem.* **1997**, *62*, 514.

17. Tani, M.; Ariyasu, T.; Nishiyama, C.; Hagiwara, H.; Watanabe, T.; Yokoyama, Y.; Murakami, Y. *Chem. Pharm. Bull.* **1996**, *44*, 48.

18. Wallace, R. A.; Kurtz, A. N.; Niemann, C. *Biochemistry* **1963**, *2*, 824.

19. (a) Imperiali, B.; Abeles, R. H. *Biochemistry* **1986**, *25*, 3760. (b) Breaux, E. J.; Bender, M. L. *FEBS Lett.* **1975**, *56*, 81.

20. Nabbs, B. K., Ph.D. thesis, University of Canterbury, 1999.

21. Le Nguyen, D.; Castro, B. *Peptide Chemistry 1987*; Protein Research Foundation: Osaka, 1988.

22. Noss, L.; Liddell, P. A.; Moore, A. L.; Moore, T. A.; Gust, D. *J. Phys. Chem. B* **1997**, *101*, 458.

23. Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gordon, E. M. *J. Med. Chem.* **1994**, *37*, 1233.

24. Zehavi, U.; Patchornik, A. *J. Am. Chem. Soc.* **1973**, *95*, 5673.

25. Kuster, G. J.; Scheeren, H. W. *Tetrahedron Lett.* **1998**, *39*, 3613.

26. Simmons, H. E.; Vest, R. D.; Vladuchick, S. A.; Webster, O. W. *J. Org. Chem.* **1980**, *45*, 5113.

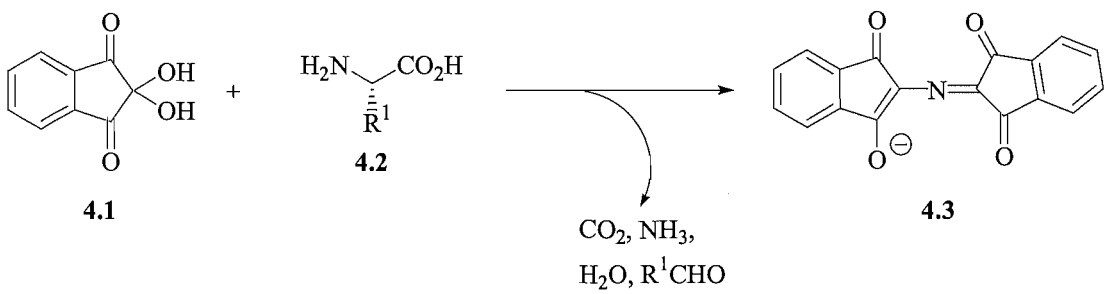
27 Abell, A. D.; Brandt, M.; Levy, M. A.; Holt, D. A. *J. Chem. Soc., Perkin Trans. 1* **1997**, 1663.

CHAPTER FOUR

DEVELOPMENT OF AN AMINO ACID *N*-DERIVATISING AGENT THAT CAN BE COLOURED ON DEMAND

4.1 INTRODUCTION

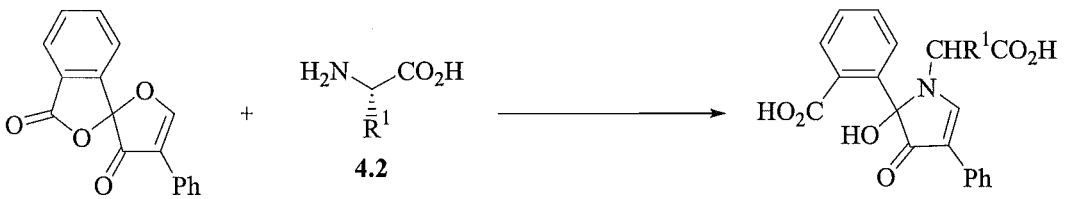
Ultraviolet-visible (UV-vis) spectroscopy is a powerful analytical technique with a wide variety of applications in chemistry, physics and biology.[1] Quantitative UV-vis spectroscopy is typically concerned with the determination of an analytes concentration,[2] whereas qualitative UV-vis analysis identifies a molecule of interest by recognition of its characteristic spectrum.[3] Amino acids, the building blocks from which proteins and polypeptides are derived, can be detected by UV-vis spectroscopy,[4] and even the nature of the variable group at the α-carbon (denoted as R^1, see Scheme 4.1) can be determined. However, direct detection of an amino acid by UV-vis spectroscopy is problematic. The functional groups common to all amino acids (*i.e.* the amine and carboxylic acid moieties) exhibit little or no absorption of UV-vis light above 210 nm, which is the wavelength range characteristically used in UV-vis spectroscopy. Some influence is exerted on UV-vis absorption by R^1, but it is dependent on the chemical nature of this group. Amino acids with an aliphatic R^1 group do not absorb light in the UV region above 220 nm (with the exception of cysteine), whereas the aromatic residues phenylalanine, tyrosine, tryptophan and histidine typically absorb between 250-300 nm.[5] The lack of activity means alternative methods such as chemical derivatisation are necessary for the reliable characterisation of amino acids by UV-vis spectroscopy. Ninhydrin (**4.1**, Scheme 4.1) is the most commonly used colourimetric assay for amino acids.[4b] Reaction of **4.1** with an amino acid (represented by **4.2**) forms the coloured compound **4.3** ("Ruhemann's purple")



Scheme 4.1. Schematic representation of the reaction between ninhydrin (**4.1**) and an amino acid (**4.2**) to give Ruhemann's purple (**4.3**).

through a complex series of intermediates.[6] Adduct **4.3** has an absorbance maximum (λ_{max}) at approximately 570 nm, and its intrinsic colour is not constant. This allows for amino acid diagnosis from the colour yield of **4.3** relative to that of leucine (arbitrarily assigned as 1.00). For example, tryptophan gives a low colour yield of 0.94, whereas the yield obtained from lysine (1.10) is more intense than that of leucine.

Fluorimetry is another technique that allows for the detection of amino acids by direct chemical modification. While several fluorometric agents exist for this purpose, the example of fluorescamine[4b] (**4.4**, Scheme 4.2) illustrates the general principles. Reaction of **4.4** with the representative amino acid **4.2** gives the highly fluorescent pyrrolinone **4.5**, which is detected fluorometrically. In a manner analogous to ninhydrin, amino acid assignment is based on the colour yield of **4.5**, relative to that derived from the reaction of **4.4** and norleucine (or glycine amide for the basic amino acids).



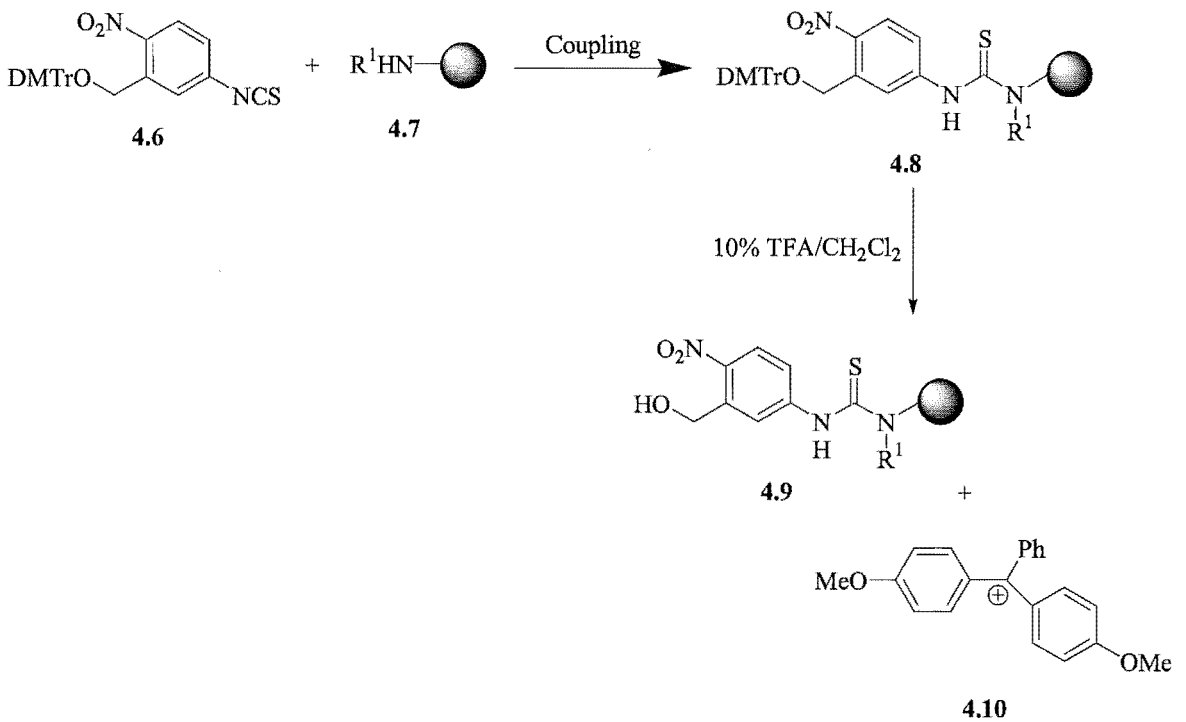
4.4 **4.2** **4.5**

Scheme 4.2. Schematic representation of the reaction between fluorescamine (**4.4**) and an amino acid (**4.2**) to give **4.5**.

The development of detection methods for amines on a solid phase support has allowed for reaction efficiency to be monitored. Formation of a peptide bond between a resin-bound amine and the carboxylic acid functionality of an *N*-protected amino acid is one reaction process that has undergone close scrutiny.[7] The coupling step needs to be reliable, fast, and proceed to almost quantitative yield, and dependable monitoring systems are required to determine the outcome of a reaction. Many of these detection systems operate by the exposure of unreacted resin-bound amino groups by reaction of the amine and a reporter molecule, which affords a brightly coloured complex. Examples of the direct modification and visualisation of resin-bound amines include the ninhydrin-based Kaiser test,[8] the *p*-

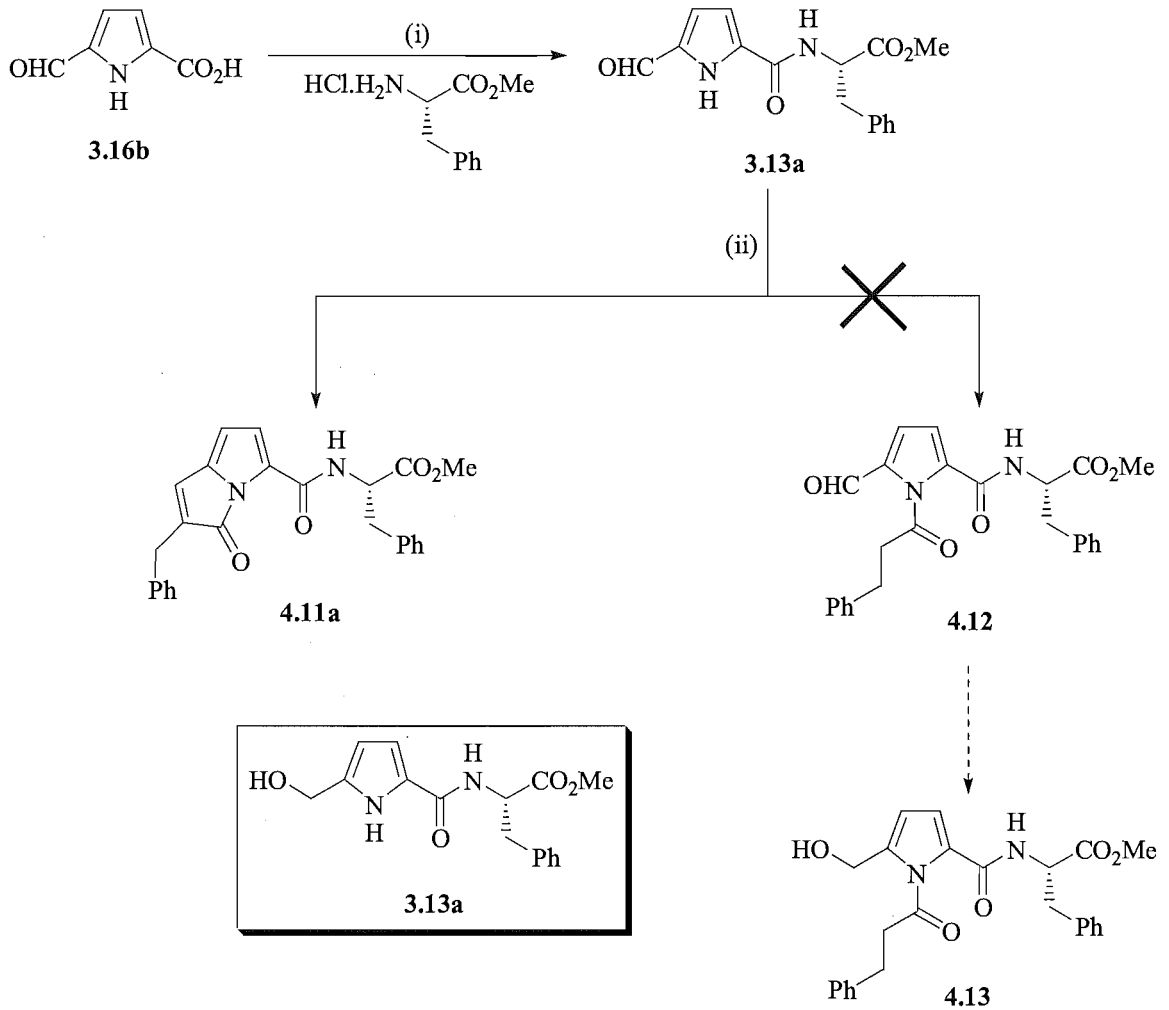
nitrophenyl ester NF31,[7] and malachite green isothiocyanate[9] for primary amines, and chloranil/acetaldehyde[10] for secondary amines.

The derivatisation of a resin-bound amino group, and subsequent diagnosis of its presence, has been divided into separate steps by Reich *et al.*[11] Reaction of nitrophenylisothiocyanate-*O*-trityl (NPIT, **4.6**, Scheme 4.3) with a resin-bound amine (**4.7**) affords adduct **4.8**. Exposure to TFA releases the latent reactivity of **4.8** to give the red coloured derivatised amine **4.9** and the dimethoxytrityl cation **4.10**. Cation **4.10** has an extinction coefficient (ε) of 76,000 M^{-1} cm^{-1} at 498 nm, which allows for the quantitative measurement of the amount of free amine present on the solid support. Qualitative detection of the amino groups is based on visual detection of **4.9**. The methodology presented in Scheme 4.3 is considered an application of modulated chemical reactivity (see Chapter One, Section 1.1 for discussion of this concept), whereby **4.10** was released on demand from a species (**4.8**) which possessed latent reactivity.



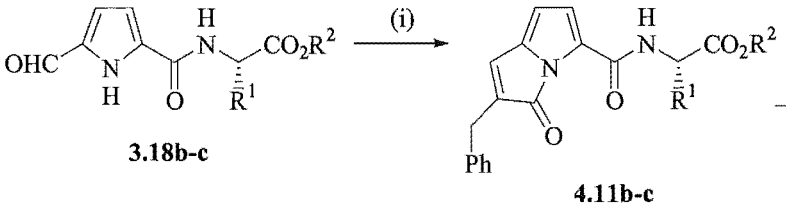
Scheme 4.3. Reaction of NPIT (**4.6**) with a resin-bound amine (**4.7**).

Despite these known techniques, there remains a need for new improved methods that reveal the presence of functional groups, such as the amine functionality of an amino acid, in a simple and reliable way.[7] Previous work in these laboratories[12] serendipitously discovered a cyclisation reaction that occurred when an essentially colourless 2,5-disubstituted pyrrole, linked by its 2-acyl substituent to an *O*-protected amino acid, was reacted with hydrocinnamoyl chloride to produce a brightly coloured pyrrolizinone. We postulate that this may form the basis of a novel method for determining the presence of amino acids. Scheme 4.4 illustrates the two-step synthetic methodology that results in a derivatised amino acid with interesting spectrophotometric properties. Reaction of **3.16b** with L-phenylalanine methyl ester hydrochloride under standard EDCI/HOBT conditions gave the *N*-derivatised amino acid **3.18a** in 91% yield. Subsequent reaction of **3.18a** with hydrocinnamoyl chloride, in the presence of DIEA and a catalytic amount of DMAP, afforded pyrrolizinone **4.11a** in 58% yield, rather than the desired intermediate **4.12**. It was hoped that **4.12** would give rise to **4.13**, a putative α-chymotrypsin inhibitor. Scheme 4.4 also displays the structure of the α-chymotrypsin inhibitor **3.13a**, prepared in Chapter Three, for comparison to **4.13**.



Scheme 4.4. *Reagents and conditions:* (i) EDCI, HOBT, DIEA, CH_2Cl_2, r.t., 16 h (91%). (ii) $Ph(CH_2)_2COCl$, DIEA, DMAP, CH_2Cl_2, r.t., 24 h (58%).[12]

The scope of this synthetic sequence was demonstrated by the preparation of two further examples.[12] The 5-formylpyrroles **3.18b-c**, derived from the separate reaction of **3.16b** with the *O*-protected amino acids L-leucine methyl ester hydrochloride and glycine ethyl ester hydrochloride, afforded **4.11b-c** in 55% and 58% yields respectively upon reaction with hydrocinnamoyl chloride (Scheme 4.5).



3.18b-c **4.11b-c**

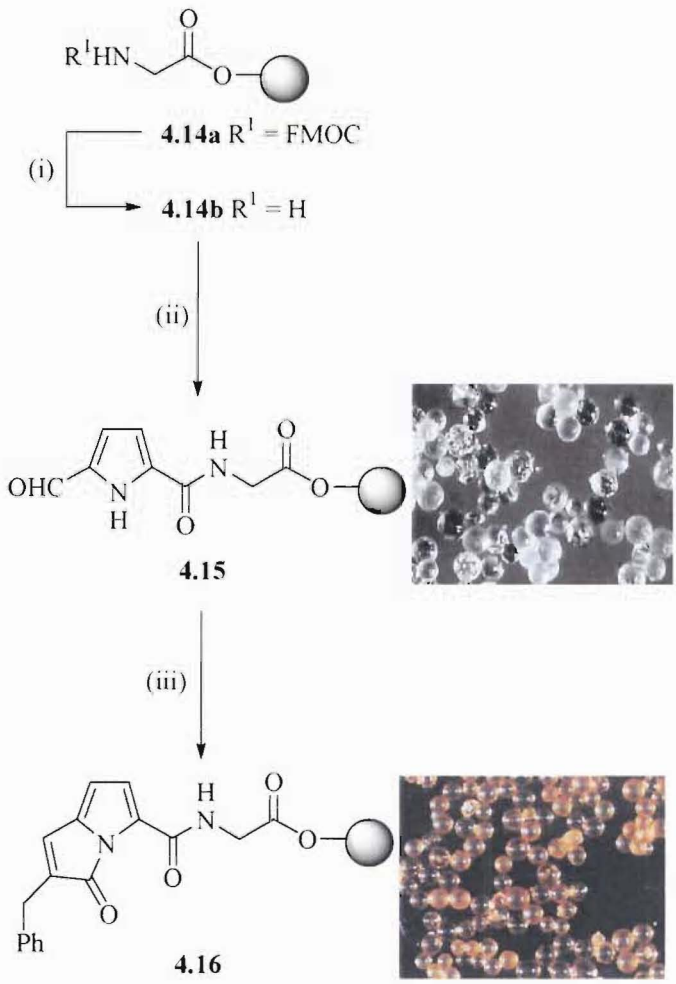
	R^1	R^2
3.18b/4.11b	CH_2CHMe_2	Me
3.18c/4.11c	H	Et

Scheme 4.5. *Reagents and conditions:* (i) $Ph(CH_2)_2COCl$, DIEA, DMAP, CH_2Cl_2, r.t., 24 h (**4.11b**, 55%), (**4.11c**, 58%).[12]

A comparison can be drawn between the reaction of ninhydrin (**4.1**, Scheme 4.1) and fluorescamine (**4.4**, Scheme 4.2) with amino acids, and preparation of the pyrrolizinones **4.11a-c** (Schemes 4.4 and 4.5). In all cases, the amine functionality of an amino acid is derivatised by chemical modification to afford products with interesting spectrophotometric properties. However, both **4.1** and **4.4** react with amino acids in a one step process, after which visualisation is performed. Preparation of the pyrrolizinones **4.11a-c** differed in that chemical modification of the amino acid was separated into two steps before the bright colouring was revealed. The amine functionality was initially "capped" with the pyrrole acid **3.16b** to give **3.18a-c**, which possess the ability to be coloured and hence visualised on demand. Upon reaction with hydrocinnamoyl chloride the spectrophotometrically active moiety was released. This methodology is considered an example of the modulation of chemical reactivity (see Chapter One, Section 1.1 for discussion of this concept), as striking spectrophotometric properties are liberated upon release of the latent reactivity possessed by the pyrrole-amino acid adducts.

This mechanism of action has relevance to the detection of amino groups in solid phase chemistry. To this end, additional work in these laboratories[13] demonstrated that the synthetic methodology utilised in Schemes 4.4 and 4.5 to effect pyrrolizinone formation was applicable to resin-bound amino acids (Scheme 4.6). Deprotection of *N*-(9-fluorenylmethoxycarbonyl)glycine Wang resin (**4.14a**) with 1:4 piperidine/dichloromethane gave the free amine **4.14b**. The pyrrole acid **3.16b** was coupled to **4.14b** by standard EDCI/HOBT methodology to afford the colourless beads **4.15**. Reaction with hydrocinnamoyl chloride gave the brightly coloured beads **4.16**, *i.e.* the presence of the

resin-bound amino groups (**4.14b**) was revealed by colour formation after two separate synthetic steps.



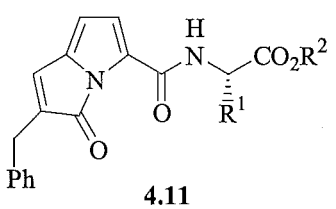
Scheme 4.6: *Reagents and conditions:* (i) 1:4 piperidine/CH_2Cl_2, r.t., 30 min. (ii) **3.16b**, EDCI, HOBT, DIEA, CH_2Cl_2, r.t., 2 h. (iii) $Ph(CH_2)_2COCl$, DIEA, DMAP, CH_2Cl_2, r.t., 5 h.[13]

The following aspects were investigated in this chapter. First, a range of pyrrolizinones were prepared to elucidate whether any differences, dictated by the variations introduced at the R^1 and *O*-protecting (R^2) groups, were observable for the λ_{max} and ε values in the UV-vis spectra of the synthesised analogues. This would demonstrate whether derivatisation of the *N*-terminal of amino acids by pyrrolizinone formation is a viable method for amino acid determination by UV-vis spectroscopy *c.f.* ninhydrin (**4.1**). Also, Beers Law would

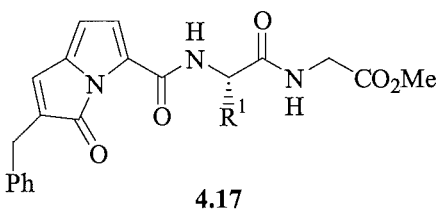
allow amino acid concentration to be determined, if the ε value was known. Inconsistencies were observed for the λ_{max} values of **4.11a-c** when methanol was used during preliminary UV-vis spectroscopy,[12] therefore additional work involved determining a solvent suitable for analysis of the pyrrolizinones. Finally, the fragments generated from the pyrrolizinones during mass spectrometry were analysed to elucidate whether R^1 could be determined by a diagnostic product ion.

4.2 SYNTHESIS OF PYRROLIZINONES WITH VARIABLE GROUPS AT R^1 AND R^2

A range of the pyrrolizinone-amino acid adducts based on the general structure **4.11** (Figure 4.1), with variability at R^1 and R^2, were synthesised for UV-vis and mass spectrometry analysis. Additionally, two dipeptidic examples based on general structure **4.17** were synthesised to determine the effect of an additional glycine residue on the UV-vis and mass spectra of the resulting pyrrolizinone-dipeptide adducts.



4.11



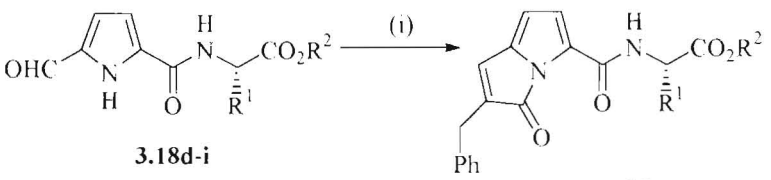
4.17

Figure 4.1. General structure of the synthetic targets **4.11** and **4.17**.

4.2.1 Synthesis of the pyrrolizinones 4.11d-i and 4.17a-b

Compounds **3.18d-i** were separately reacted with hydrocinnamoyl chloride to give the pyrrolizinones **4.11d-i** in yields of 42%, 35%, 31%, 44%, 21% and 43% respectively

(Scheme 4.7). It is important to note that strictly anhydrous reaction conditions were needed for pyrrolizinone formation to occur.



	R^1	R^2
3.18d/4.11d	H	Bn
3.18e/4.11e	CH_2Ph	Et
3.18f/4.11f	CH_2CHMe_2	Et
3.18g/4.11g	$CHMe_2$	Me
3.18h/4.11h	Me	Me
3.18i/4.11i	H	Me

Scheme 4.7. *Reagents and conditions:* (i) $Ph(CH_2)_2COCl$, DIEA, DMAP, CH_2Cl_2, r.t., 24 h (**4.11d**, 42%), (**4.11e**, 35%), (**4.11f**, 31%), (**4.11g**, 44%), (**4.11h**, 21%), (**4.11i**, 43%).

A single crystal X-ray structure of **4.11h** (Figure 4.2) was determined for comparison to reported crystal structures of pyrrolizin-3-ones, and to characterise the extended conjugated backbone of this molecule. This compound crystallised, with an orthorhombic space group *I*222, from an ethyl acetate/petroleum ether solvent system by the technique of vapour diffusion (see Appendix for crystal structure details).

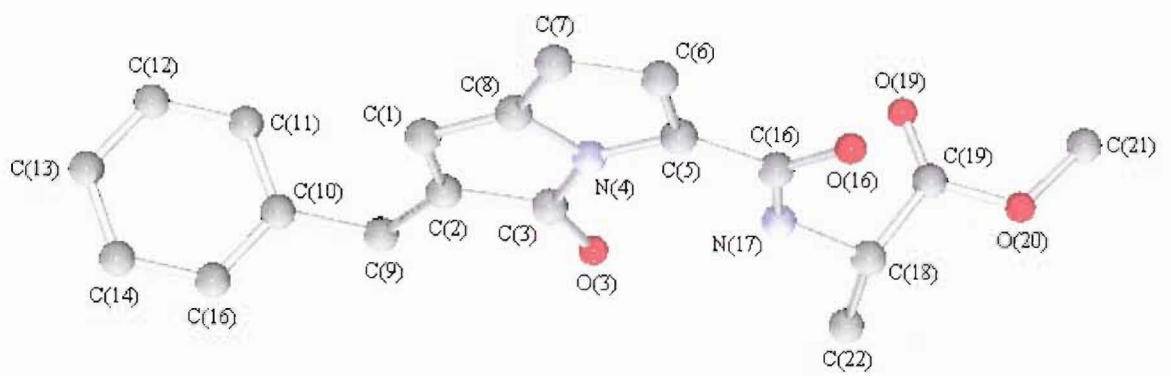
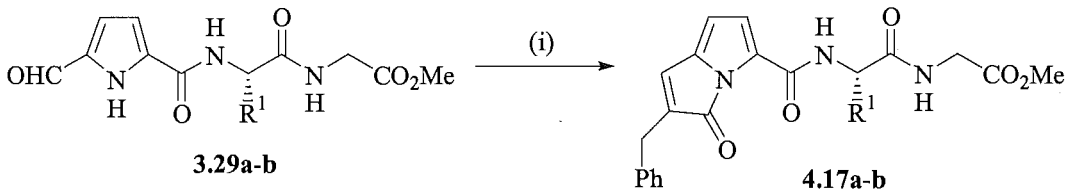


Figure 4.2. X-ray crystal structure of **4.11h** with crystallographic numbering system.

Analysis of the crystal structure of **4.11h** illustrated that it possessed the structural characteristics observed in previous reports.[12,14] The bicyclic ring system is essentially planar, and the amide bonds lengths are noticeably different to those of a normal cyclic tertiary amide.[15] Compound **4.11h** has a C(3)-N(4) bond length of 1.413(3) Å, which is longer than the C-N bond length (1.335(9) Å) of a normal cyclic tertiary amide. In contrast, the C(3)-O(3) bond, at 1.214(3) Å, is somewhat shorter than that of a C=O bond

in a normal cyclic tertiary amide (typically 1.234(11) Å). This observation is consistent with the reduced amide bond delocalisation previously reported for pyrrolizin-3-ones.[12,14] The altered bond lengths of the pyrrole substructure of **4.11h** compared to pyrrole[16] itself further supported the idea of reduced delocalisation in the pyrrolizin-3-one ring system compared to pyrrole itself.

Scheme 4.8 outlines the synthetic route towards pyrrolizinone-dipeptide adducts of type **4.17**. Reaction of **3.29a-b** with hydrocinnamoyl chloride afforded the dipeptide pyrrolizinones **4.17a-b** in yields of 34% and 33% respectively (Scheme 4.9).



3.29a-b **4.17a-b**

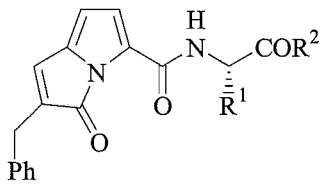
	R^1
3.29a/4.17a	CH_2Ph
3.29b/4.17b	CH_2CHMe_2

Scheme 4.8: *Reagents and conditions:* (i) $Ph(CH_2)_2COCl$, DIEA, DMAP, CH_2Cl_2, r.t., 24 h (**4.17a**, 34%), (**4.17b**, 33%).

4.3 INITIAL UV-VIS SPECTROSCOPIC ANALYSIS OF THE PYRROLIZINONE 4.11e USING METHANOL AS SOLVENT

The initial goal of the spectroscopic studies reported in this chapter was to give a deeper understanding of the UV-vis spectrophotometric properties possessed by pyrrolizinones of type **4.11/4.17**. Specifically, the influence that R^1 and R^2 had upon the λ_{max} and ε values in the UV-vis spectra recorded for compounds of type **4.11/4.17** was to be determined. Any conspicuous trends would form the basis of a technique that would allow elucidation of the R^1 and R^2 groups by *N*-derivatisation of an amino acid, with subsequent analysis by UV-

vis spectroscopy. Also, once the ε value is known, the concentration of an amino acid in a mixture could be quantified by Beers Law. Pyrrolizinones **4.11a-c** were subjected to UV-vis spectroscopic analysis in the previous study,[12] however noticeable differences were observed in the λ_{max} values obtained for these compounds. These differences were dependent upon the solvent used for UV-vis spectroscopy. The spectrum for **4.11a**, obtained in chloroform, possessed two maxima at 301 and 438 nm (Table 4.1). In contrast, **4.11b** and **4.11c** were analysed in methanol, and both pyrrolizinones had three λ_{max} values.

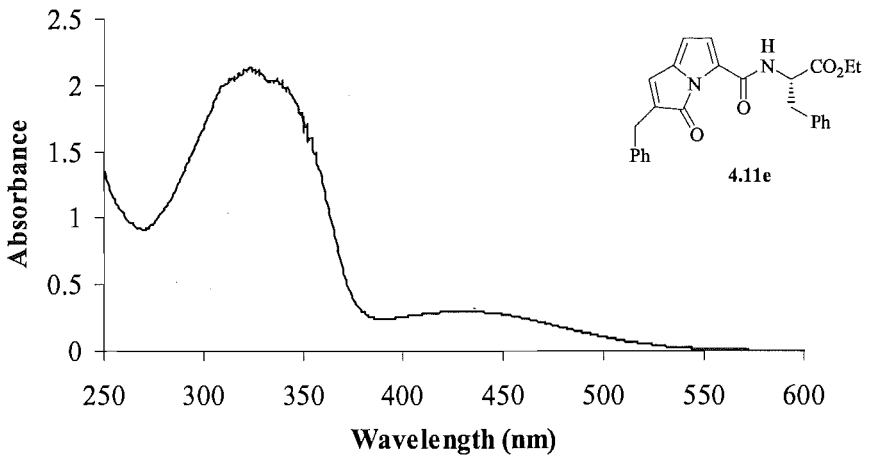


No.	R^1	R^2	Solvent	λ_{max} (nm) [ε (M^{-1} cm^{-1})]
4.11a	CH_2Ph	OMe	$CHCl_3$	301 [12,300], 438 [4,100]
4.11b	CH_2CHMe_2	OMe	MeOH	223 [11,500], 339 [9,100], 436 [900]
4.11c	H	OEt	MeOH	242 [11,300], 338 [25,400], 423 [200]

Table 4.1. UV-vis spectroscopic data for the pyrrolizinones **4.11a-c**.[12]

A time-course study was implemented to determine what effect the use of a nucleophilic solvent such as methanol had upon a representative pyrrolizinone during UV-vis spectroscopy. To this end, **4.11e** was dissolved in methanol and allowed to stand at r.t. under an ambient atmosphere. UV-vis spectra of this solution were taken at 10 min intervals. At 0 min a spectrum was obtained with two λ_{max} at approximately 330 and 430 nm (Figure 4.3a). After 30 min the shape of the absorbance plot had changed markedly (Figure 4.3a). The λ_{max} at approximately 430 nm had disappeared, and the λ_{max} at approximately 330 nm was replaced by an extremely distorted signal between 310-360 nm. This result revealed that pyrrolizinones such as **4.11e** were unstable in methanol, and therefore it is an unsuitable solvent for UV-vis spectroscopy.

(a)



(b)

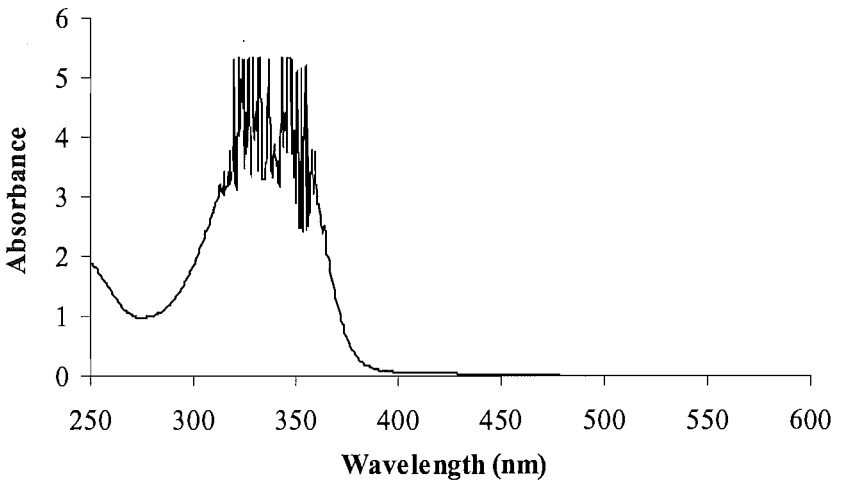
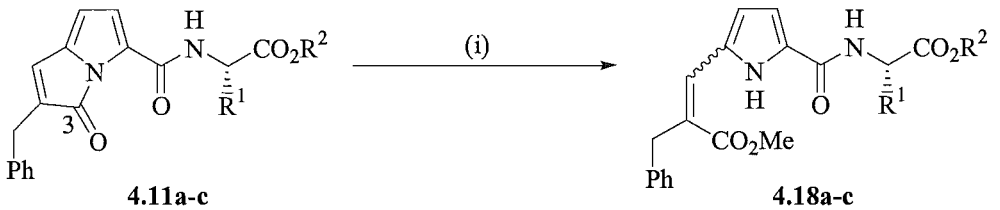


Figure 4.3. UV-vis spectra of **4.11e** after 0 min (a) and 30 min (b).

The reactivity of the pyrrolizinone ring system upon exposure to nucleophiles has been demonstrated previously.[12] Pyrrolizones **4.11a-c** were reacted with 15 equivalents of sodium methoxide in methanol to give the colourless pyrrole acrylic esters **4.18a-c** within 1 min, in yields of 85%, 82% and 80% respectively (Scheme 4.9). This sequence provides a method for "decolourising" the amino acid, hence the spectrophotometric properties of the pyrrolizinone can be subsequently removed at a desired stage.



4.11a-c **4.18a-c**

	R^1	R^2
4.11a/4.18a	CH_2Ph	Me
4.11b/4.18b	CH_2CHMe_2	Me
4.11c/4.18c	H	Et

Scheme 4.9: *Reagents and conditions:* (i) MeONa, MeOH, r.t., 1 min (**4.18a**, 82%), (**4.18b**, 80%), (**4.18c**, 85%).[12]

An identical process, *i.e.* ring opening of the pyrrolizinone at C3, must have occurred when **4.11e** was allowed to stand in methanol for 30 min. To avoid pyrrolizinone degradation during UV-vis spectroscopy, it was deemed prudent to exchange methanol for the relatively inert solvent acetonitrile.

4.4 UV-VIS SPECTROSCOPIC ANALYSIS OF THE PYRROLES 3.18e AND 3.18i, AND THE PYRROLIZINONES 4.11a-i AND 4.17a-b

Determining the influence of R^1 and R^2 on the UV-vis spectra recorded for compounds of type **4.11/4.17** in acetonitrile was then undertaken. However, preliminary UV-vis spectroscopy was performed on the pyrroles **3.18e** and **3.18i** in order to determine the activity exhibited by the pyrrole-based precursors. This was carried out to establish to what extent the activity demonstrated by the pyrrolizinones was dependent on the pyrrole-amino acid substructure. Compound **3.18e** was chosen as an example of a precursor with a UV active aromatic group at R^1, and **3.18i** as one with a non-aromatic side chain. Figure 4.4 displays the UV-vis spectrum for **3.18e**, which was found to have a λ_{max} of 291 nm (ε = 28,610 M^{-1} cm^{-1}).

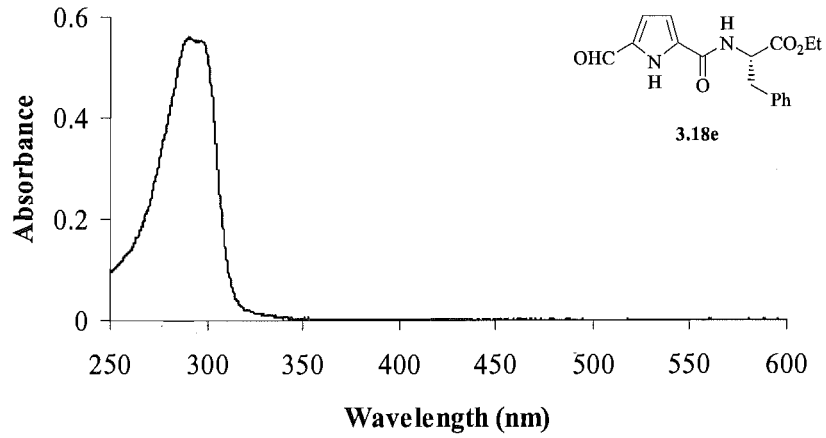


Figure 4.4. UV-vis spectrum for **3.18e**.

The λ_{max} of 289 nm observed for **3.18i** (Figure 4.5) was almost identical to that obtained for **3.18e**, but the ε value calculated for **3.18i** was much lower (16,780 M^{-1} cm^{-1}). These results indicated that compounds of type **3.18** have λ_{max} of approximately 290 nm, but the ε value appeared to be dependent on the nature of the side chain. The almost identical λ_{max} values for **3.18e** and **3.18i** was attributed to the conjugated nature of the 2,5-disubstituted pyrrole ring system possessed by both compounds.

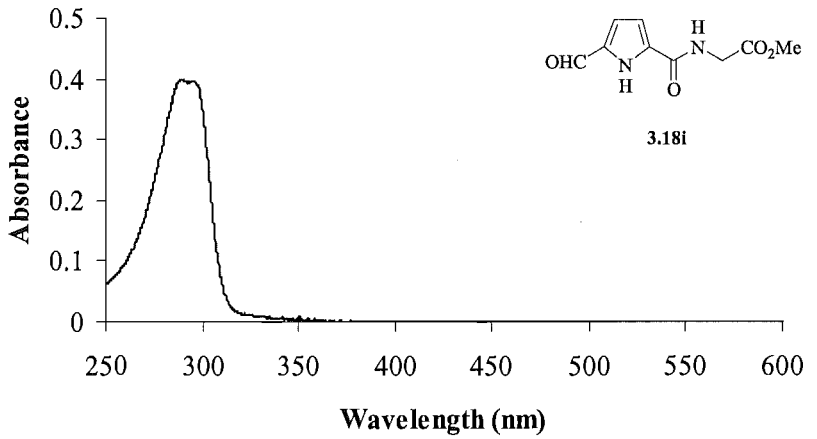


Figure 4.5. UV-vis spectrum for **3.18i**.

UV-vis spectroscopic analysis of the pyrrolizinones **4.11a-i** and **4.17a-b** was subsequently performed to determine what influence R^1 and R^2 had upon the λ_{max} and ε values.

Accurate comparisons between **4.11a-i** and **4.17a-b** required analysis of the previously synthesised compounds **4.11a-c**.[12] The spectral plot of **4.11d**, displayed in Figure 4.6, is typical for all pyrrolizinones.

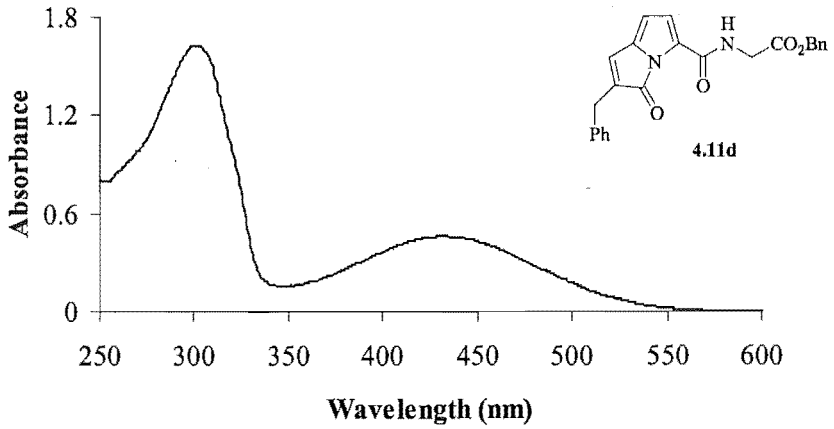
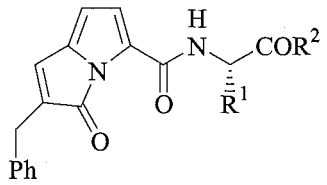


Figure 4.6. UV-vis spectrum for **4.11d**.

Table 4.2 presents the UV-vis spectroscopic data for compounds **4.11a-i** and **4.17a-b** in freshly distilled acetonitrile. Data for **3.18e** and **3.18i**, the synthetic precursors to **4.11e** and **4.11i** respectively, is also displayed for comparison.



No.	R^1	R^2	λ_{max} (nm) [ε (M^{-1} cm^{-1})]	Ratio $\varepsilon_{(\sim 300\ nm)}/\varepsilon_{(\sim 430\ nm)}$
4.11a[#]	CH_2Ph	OMe	299 [6380], 434 [1950]	3.27
4.11b[#]	CH_2CHMe_2	OMe	300 [5010], 434 [1550]	3.23
4.11c[#]	H	OEt	299 [8200], 433 [2150]	3.81
4.11d	H	OBn	299 [5610], 431 [1530]	3.67
4.11e	CH_2Ph	OEt	299 [8500], 432 [1920]	4.45
(3.18e)	CH_2Ph	OEt	291 [28610]	na*
4.11f	CH_2CHMe_2	OEt	298 [7450], 429 [1580]	4.71
4.11g	$CHMe_2$	OMe	300 [7580], 434 [1780]	4.25
4.11h	Me	OMe	299 [8950], 434 [2190]	4.09
4.11i	H	OMe	297 [7190], 433 [1860]	3.86
(3.18i)	H	OMe	289 [16780]	na*
4.17a	CH_2Ph	$NHCH_2CO_2Me$	299 [5500], 432 [1570]	3.50
4.17b	CH_2CHMe_2	$NHCH_2CO_2Me$	298 [7100], 435 [1840]	3.86

[#] = Previously synthesised[12]
* = Not applicable

Table 4.2. UV-vis spectral data for pyrrolizinones **4.11a-i** and **4.17a-b** in acetonitrile. Data for **3.18e** and **3.18i** is included underneath the respective pyrrolizinone analogues **4.11e** and **4.11i** for comparison.

The distinctive shape of the spectral plots for pyrrolizinones of type **4.11/4.17** (see Figure 4.6) arose from splitting of the single λ_{max} possessed by pyrroles of type **3.18** at approximately 290 nm. As expected, the more extensive conjugation of the pyrrolizinone ring system, compared to the pyrrole-based structures, resulted in the two λ_{max} possessed by compounds of type **4.11/4.17** absorbing at wavelengths longer than 290 nm.[17] The spectra of **4.11a-i** and **4.17a-b** were then compared to determine whether the variable R^1

and R^2 groups had any effect on the λ_{max} or ε values. All of the pyrrolizinones have λ_{max} at approximately 300 and 430 nm, eliminating λ_{max} as a method of identifying R^1 or R^2. Comparison of the ε values for **4.11a-i** and **4.17a-b** at either approximately 300 or 430 nm also revealed they were not dependent on the presence of a specific R^1 or R^2 group.

Further analysis of the data in Table 4.2 was performed in order to ascertain any influence on the ε values that resulted from the nature of R^1 or R^2. A ratio was calculated by division of the ε value at approximately 300 nm by the ε value at approximately 430 nm (*i.e.* $\varepsilon_{(\sim 300\ nm)}/\varepsilon_{(\sim 430\ nm)}$), and the ratios for **4.11a-i** and **4.17a-b** appear in the right-hand column of Table 4.2. However, there was no correlation between the ratio and the nature of the group at R^1 or R^2. For example, comparison of the ratios obtained for compounds that possessed an identical R^1 group gave noticeably different values. For example, **4.11c, 4.11d** and **4.11i** (R^1 = H) gave relatively similar values of 3.81, 3.67 and 3.86 respectively, whereas **4.11b**, **4.11f** and **4.17b** (R^1 = CH_2CHMe_2) gave disparate ratios of 3.23, 4.71 and 3.86 respectively. In contrast, a comparison of two dissimilar R^1 groups gave little variation in the calculated ratios, *e.g.* **4.11a** (R^1 = CH_2Ph) had a ratio of 3.27, which was almost identical to the ratio of 3.23 for **4.11b** (R^1 = CH_2CHMe_2). The R^2 group also had little influence over the value for $\varepsilon_{(\sim 300\ nm)}/\varepsilon_{(\sim 430\ nm)}$. Pyrrolizinones with identical R^2 functionalities gave dissimilar ratios, *e.g.* the low value of 3.27 obtained for **4.11a** (R^2 = OMe) versus the high ratio of 4.25 obtained for **4.11g** (R^2 = OMe). In contrast, similar R^2 groups gave almost identical ratios, *e.g.* the values of 3.27 and 3.23 given by **4.11a** (R^2 = OMe) and **4.11b** (R^2 = OMe) respectively. Overall, the lack of recurring trends for R^1 and R^2, by inspection of the λ_{max}, ε or $\varepsilon_{(\sim 300\ nm)}/\varepsilon_{(\sim 430\ nm)}$ values, meant that UV-vis spectroscopy did not allow for the identification of what functionality was present at these positions.

However, then derivatisation of an amino acid to give a compound of type **4.11** becomes a method for quantifying the amount of an unknown amino acid by the use of Beers Law (Figure 4.7), and the reference data in Table 4.2, to calculate pyrrolizinone concentration.

$$A = \varepsilon c l$$

Figure 4.7. Beers Law (A = absorbance, ε = extinction coefficient, c = concentration, l = sample path length).

4.5 MASS SPECTROMETRY FRAGMENTATION ANALYSIS OF THE PYRROLIZINONES 4.11a-i AND 4.17a-b

The LRMS spectra of **4.11a-i** and **4.17a-b** were analysed to determine whether a product ion was generated during mass spectrometry whose *m/z* ratio was dictated by R^1. This would form the basis of a technique whereby a particular amino acid might be diagnosed by derivatisation and subsequent mass spectrometry. Two features were revealed, the first of which was the presence of four product ions in all spectra. The most noticeable was the base peak at *m/z* 236.1, which is formed by fragmentation of the molecular ion ($M^{+\cdot}$) of the representative pyrrolizinone **4.11** to give product ion **4.19** (Figure 4.8). The molecular formula of **4.19** ($C_{15}H_{10}NO_2^{\cdot}$) was confirmed by analysis of the HRMS data. Another ubi-

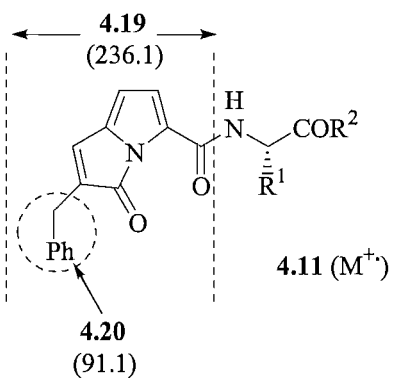
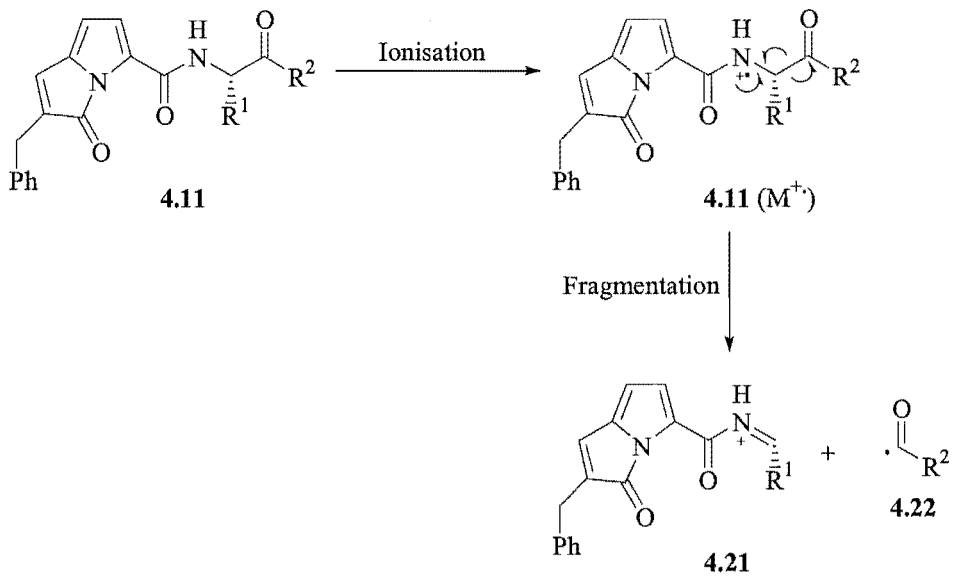


Figure 4.8. Fragmentation of the pyrrolizinones represented **4.11** afforded the product ions **4.19** and **4.20**.

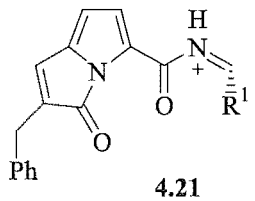
quitous fragment had *m/z* of 91.1, which corresponded to the benzyl product ion **4.20** (HRMS data confirmed a molecular formula of $C_7H_7^+$). The abundance of **4.20** in the spectra of **4.11a-i** and **4.17a-b** ranged between 5-29%, relative to **4.19**, and its formation is favoured due to resonance stabilisation.[18] Two other ubiquitous fragments had *m/z* of 153.1 (8-17% abundance relative to **4.19**) and 180.1 (5-14% abundance relative to **4.19**). However, analysis of the HRMS data for these two product ions did not lead to any definitive structural assignment.

The second and most important feature of the fragmentation patterns was a product ion that revealed the nature of R^1. Analysis of the LRMS data indicated that cleavage of the bond between the α-carbon of the amino acid contiguous to the pyrrolizinone ring system, and the carbonyl carbon of the ester or amide functionality, produced a product ion that revealed the structure of R^1. Cleavage at this point is typical of the amino acids.[19] The postulated mechanism of cleavage for compounds of type **4.11** is illustrated in Scheme 4.10. Removal of one electron from the amide nitrogen of **4.11** results in conversion to the molecular ion, which undergoes α-cleavage[19] to give the product ions **4.21** and **4.22**.



Scheme 4.10. Postulated fragmentation mechanism for the molecular ion **4.11** ($M^{+\cdot}$), to give the product ions **4.21** and **4.22**.

Table 4.3 summarises the information given by examination of the fragmentation patterns observed in the LRMS spectra of **4.11a-i** and **4.17a-b**. All compounds were subjected to electron impact ionisation mass spectrometry.



4.21

No.	R^1	*m/z* of $M^{+\cdot}$	*m/z* of ion **4.21** (% intensity)	$M^{+\cdot}$ - (*m/z* **4.21**)	$M^{+\cdot}$ - (*m/z* **4.21**) corresponds to loss of:
4.11a[#]	CH_2Ph	414.2	355.1 (6)	59.1	$-CO_2Me$
4.11b[#]	CH_2CHMe_2	380.2	321.2 (31)	59.0	$-CO_2Me$
4.11c[#]	H	338.1	265.1 (7)	73.0	$-CO_2Et$
4.11d	H	400.1	265.1 (8)	135.0	$-CO_2CH_2Ph$
4.11e	CH_2Ph	428.2	355.1 (7)	73.1	$-CO_2Et$
4.11f	CH_2CHMe_2	394.2	321.2 (76)	73.0	$-CO_2Et$
4.11g	$CHMe_2$	366.2	307.1 (46)	59.1	$-CO_2Me$
4.11h	Me	338.1	279.1 (40)	59.0	$-CO_2Me$
4.11i	H	324.1	265.1 (8)	59.0	$-CO_2Me$
4.17a	CH_2Ph	471.2	355.2 (42)	116.0	$-CONHCH_2CO_2Me$
4.17b	CH_2CHMe_2	437.2	321.2 (81)	116.0	$-CONHCH_2CO_2Me$

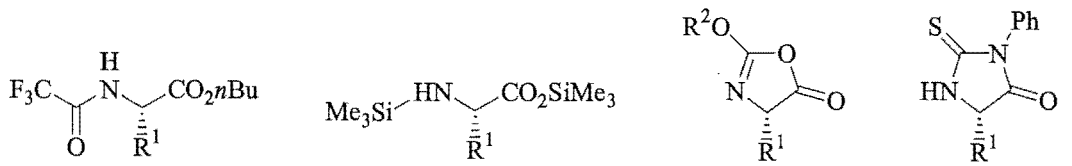
[#] = Previously synthesised[12]

Table 4.3. LRMS data for pyrrolizinones **4.11a-i** and **4.17a-b**, which afforded the product ion represented by **4.21**.

Fragmentation of the pyrrolizinones **4.11a-i** and **4.17a-b** to give a product ion of type **4.21** appeared to be a reliable phenomenon for these compounds. The mass of **4.21** was dependent on the R^1 group. Almost all of the natural amino acids have a unique mass, which is dictated by R^1. The only exceptions where identical masses are given by different amino acids are glutamine and the free base of lysine, glutamic acid and protonated lysine,

and the constitutional isomers leucine and isoleucine. Hence, an amino acid can be characterised by the presence of a diagnostic product ion such as **4.21** in a mass spectrum.

Derivatisation of amino acids and subsequent analysis by mass spectrometry has previously allowed assignment of the R^1 group by the *m/z* ratios of distinctive product ions. Examples include the *N*-trifluoroacetyl amino acid *n*-butyl esters represented by **4.23**[20] (Figure 4.9), trimethylsilyl derivatives such as **4.24**,[21] oxazolones and closely related cyclic structures[19] (based on **4.25**), and the phenylthiohydantoin-based compounds represented by **4.26**.[22] Products of type **4.26** were generated by automated degradation of a protein with phenylisothiocyanate (Edman's reagent), and the polypeptide sequence was subsequently determined by chemical ionisation LRMS analysis of the resulting adducts.[22] Precedence also exists for elucidation of the R^1 group by mass spectrometry after the derivatisation of amino acids with dansyl chloride, fluorescamine (see Scheme 4.2), and a range of *N*-acyl groups.[19] All of the methods discussed here function by the direct chemical modification of an amino acid (or its ester equivalent), followed by mass spectrometric analysis. The method presented in this chapter differs in that the amino acid is derivatised in two steps, with the second step unmasking the pyrrolizinone when desired. This product undergoes fragmentation during mass spectrometry to give a product ion (**4.21**), whose *m/z* ratio is dictated by the R^1 group.



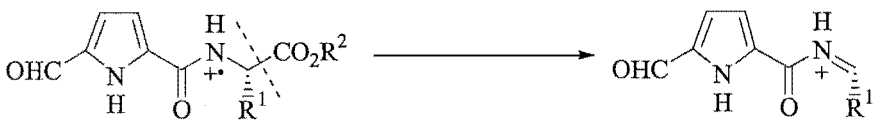
4.23 **4.24** **4.25** **4.26**

Figure 4.9. Derivatised amino acids **4.23-4.26**.

The nature of the R^1 group appeared to influence the abundance of the product ion relative to the base peak (see Table 4.3). Particularly strong intensities (between 31-76%) were observed for the product ion **4.21** derived from compounds **4.11b** and **4.11f-h** (R^1 = alkyl). This discovery was surprising, as amino acids that possess an alkyl group of two or more

carbons tend to undergo fragmentation in a way that results in cleavage of the R^1 group from the α-carbon.[19] Compounds with either R^1 = H (**4.11c**, **4.11d** and **4.11i**) or R^1 = CH_2Ph (**4.11a** and **4.11e**) had a lower abundance of the product ion (between 6-8%). A low signal intensity for the product ion derived from **4.11a** and **4.11e** was expected, as amino acids with a side chain of type CH_2Ar tend to eject the highly stable species $^{\cdot}CH_2Ar$.[19] The pyrrolizinone dipeptides **4.17a-b** gave product ions with relatively strong intensities (42% and 81% respectively), and for the compound where R^1 is an alkyl group (**4.17b**) a higher intensity of **4.21** was observed than that for **4.17a** (R^1 = CH_2Ph). Comparison of the dipeptide pyrrolizinones **4.17a-b** to their corresponding monopeptide analogues illustrated that the adjacent glycine residue generally increased the abundance of **4.21**. Fragmentation of **4.17a** (R^1 = CH_2Ph) gave a product ion with an abundance of 42%, which was much higher than that derived from its methyl ester (**4.11a**, 6%) or ethyl ester (**4.11e**, 7%) analogues. A similar trend was noticed for **4.17b** (R^1 = CH_2CHMe_2), which gave a product ion with an abundance (81%) that was greater than that derived from its methyl ester analogue **4.11b** (31%), although the ethyl ester analogue **4.11f** gave a product ion with a similar intensity (76%).

Interestingly, the 5-formylpyrroles **3.18a-i** and **3.29a-b** also displayed a fragmentation process analogous to that observed for **4.11a-i** and **4.17a-b**. Scheme 4.11 illustrates that α-cleavage of the molecular ion generated from compounds of type **3.18/3.29** gave the product ion **4.27**.

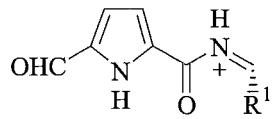


3.18/3.29 ($M^{+\cdot}$) **4.27**

Scheme 4.11. Fragmentation mechanism for the molecular ion ($M^{+\cdot}$) of type **3.18/3.29** to give the product ion **4.27**. The point of cleavage is marked by a dashed line.

Analysis of the fragmentation patterns in the LRMS spectra for **3.18a-i** and **3.29a-b** gave the data summarised in Table 4.4. All compounds were subjected to electron impact

ionisation mass spectrometry, with the exception of **3.29b**, which was analysed by electrospray ionisation mass spectrometry



4.27

No.	R^1	*m/z* of $M^{+\cdot}$	*m/z* of ion **4.27** (% intensity)	$M^{+\cdot}$ - (*m/z* **4.27**)	$M^{+\cdot}$ - (*m/z* **4.27**) corresponds to loss of:
3.18a*	CH_2Ph	300.1	241.1 (5)	59.0	$-CO_2Me$
3.18b#	CH_2CHMe_2	266.1	207.1 (38)	59.0	$-CO_2Me$
3.18c#	H	224.1	151.1 (25)	73.0	$-CO_2Et$
3.18d	H	286.1	151.1 (18)	135.0	$-CO_2CH_2Ph$
3.18e	CH_2Ph	314.1	241.1 (6)	73.0	$-CO_2Et$
3.18f	CH_2CHMe_2	280.1	207.1 (47)	73.0	$-CO_2Et$
3.18g	$CHMe_2$	252.1	193.1 (44)	59.0	$-CO_2Me$
3.18h	Me	224.1	165.0 (98)	59.1	$-CO_2Me$
3.18i	H	210.1	151.1 (25)	59.0	$-CO_2Me$
3.29a	CH_2Ph	357.1	241.1 (25)	116.0	$-CONHCH_2CO_2Me$
3.29b¶	CH_2CHMe_2	346.1‡	207.1 (12)	139.0	$-CONHCH_2CO_2Me - Na^+$

* = Synthesised by the author, but mass spectrometry results were obtained from the previous study[12]
# = Previously synthesised[12]
¶ = Analysed by electrospray ionisation LRMS
‡ = M^++Na

Table 4.4. LRMS data for pyrroles **3.18a-i** and **3.29a-b**, which afforded the product ion represented by **4.27**.

The LRMS data for the pyrroles **3.18a-i** and **3.29a-b** displayed some of the trends observed for the pyrrolizinones **4.11a-i** and **4.17a-b**. Most importantly, the mass of product ion **4.27** was dictated by R^1, which suggested that the amino acid side chain can be determined by mass spectrometry at either step of the derivatisation technique presented in this chapter.

The abundance of **4.27** appeared to be dependent on the nature of R^1. Derivatives with an alkyl R^1 group (**3.18b** and **3.18f-h**) had a greater intensity recorded for **4.27** (38%, 47%, 44% and 98% respectively), in comparison to those with $R^1 = CH_2Ph$ (**3.18a**, **3.18e** and **3.29a**, 5%, 6% and 25% respectively). Compounds where $R^1 = H$ (**3.18c-d** and **3.18i**) gave product ion intensities of 25%, 18% and 25% respectively, which were noticeably higher than those recorded for the product ion **4.21** derived from the corresponding pyrrolizinones **4.11c-d** and **4.11i** (7%, 8% and 8% respectively). This suggested that the first derivatisation step, whereby the pyrrole derivative **3.16b** was coupled to the *N*-terminus of a glycine derivative, gave an adduct that afforded greater levels of the diagnostic product ion in comparison to the resultant pyrrolizinones. The relatively low abundance of product ion derived from **3.29b** (12%) resulted from the use of electrospray ionisation, a "soft" ionisation technique that leads to lessened fragmentation of the parent ion.[18] This result indicated that electron impact ionisation was more effective than electrospray ionisation for production of the fragments of interest, **4.21** and **4.27**. Finally, the presence of an additional glycine residue at the R^2 position of the pyrrole-dipeptide adduct **3.29a** ($R^1 = CH_2Ph$) resulted in a greater abundance of **4.27** (25%), compared to that derived from the methyl ester (**3.18a**, 5%) and ethyl ester (**3.18b**, 6%) analogues. This was in agreement with what was observed for the corresponding pyrrolizinone-dipeptide adduct **4.17a**.

4.6 CONCLUSION AND FUTURE WORK

Previous work in these laboratories developed a procedure for the two-step derivatisation of amino acids,[12] which began by the attachment of a pyrrole derivative to the amino group. Subsequent reaction afforded the pyrrolizinones **4.11a-c**, which displayed a bright colouring and interesting UV spectroscopic behaviour. This two-step procedure was proposed to be a novel derivatisation method, whereby the amino functionality was initially "capped" by reaction with the pyrrole acid **3.16b**, and the latent reactivity of the attached pyrrole derivative was released under mild reaction conditions to reveal the presence of an amino group by pyrrolizinone formation.

Expansion of the range of amino acids reacted under these conditions, which afforded the pyrrolizinones **4.11d-i** and **4.17a-b**, demonstrated that the introduction of variable groups at R^1 and R^2 gave no observable differences in the UV spectra. However, this derivatisation method could be used to quantify the amount of an amino acid by the use of Beers Law. The use of methanol was not compatible with the UV-vis spectroscopic analysis of compounds of type **4.11/4.17** due to degradation of the pyrrolizinone ring structure, and acetonitrile was found to be a more suitable solvent.

Analysis of the fragmentation patterns in the LRMS spectra of **4.11a-i** and **4.17a-b** revealed that a product ion (**4.21**), which resulted from cleavage between the α-carbon of the amino acid and the adjacent carbonyl carbon, was present in all spectra. The mass of **4.21** was dependent on R^1. Additionally, the nature of R^1 dictated the abundance of **4.21** – if it was an alkyl group the intensity was higher than if R^1 = H or CH_2Ph. A glycine residue adjacent to the amino acid contiguous to the pyrrolizinone ring system increased the signal intensity of **4.21** in comparison to the monopeptide analogues with identical R^1 groups. Analysis of the fragmentation patterns for **3.18a-i** and **3.29a-b**, the synthetic precursors to the pyrrolizinones, indicated that the α-cleavage observed for the pyrrolizinones was also a facile process for the pyrrole-amino acid adducts. Both the mass and abundance of the product ion **4.27** was dictated by R^1, with signal intensity increased by the presence of an alkyl side chain group relative to those with R^1 = CH_2Ph.

Fragmentation of the pyrrole-glycine adducts **3.18c-d** and **3.18i** gave an abundance of the diagnostic product ion that was noticeably higher than what resulted from the analogous pyrrolizinones **4.11c-d** and **4.11i**. This suggested that derivatisation of glycine with the pyrrole **3.16b** gave greater levels of the diagnostic product ion. The signal intensity of **4.27**, derived from the pyrrole-dipeptide adduct **3.29b**, was lower than expected due to a reduced level of fragmentation which resulted from the use of electrospray ionisation LRMS. The technique of electron impact ionisation was therefore deemed more suitable for the elucidation of R^1, as this method is known to cause a greater level of parent ion fragmentation, and consequently more of the product ion would be observed.

Future work could involve the determination of other functional groups that have the ability to be derivatised and subsequently revealed by this methodology. Any difference in the behaviour of these novel pyrrolizinones under both UV spectroscopy and mass spectrometry, in comparison to that of the amino acid-based pyrrolizinones, would be determined. An investigation towards the potential of Beers Law as a means for determining what group was adjacent to the pyrrolizinone could also be undertaken. The application of this two-step derivatisation method to the sequencing of peptides could also be investigated.

4.7 REFERENCES FOR CHAPTER FOUR

1. Perkampus, H. H. *UV-VIS Spectroscopy and Its Applications*; Springer-Verlag: Berlin, 1992.

2. Jaffe, H. H.; Orchin, M. In *Theory and Applications of Ultraviolet Spectroscopy*; John Wiley and Sons, Inc.: New York, 1962, pp. 556-586.

3. Davies, B. In *UV Spectroscopy: Techniques, instrumentation and data handling*; Clark, B. J., Frost, T., Russell, M. A., Eds.; Chapman & Hall: London, 1993, pp. 63-77.

4. (a) Barrett, G. C.; Elmore, D. T. In *Amino Acids and Peptides*; Cambridge University Press: Cambridge, 1998, pp. 32-47. (b) Rosenthal, G. A. In *Chemistry and Biochemistry of the Amino Acids*; Barrett, G. C., Ed.; Chapman and Hall Ltd: London, 1985, pp. 573-590.

5. Kleeman, A.; Leuchtenberger, W.; Hopper, B.; Tanner, H. In *Ullmann's Encyclopedia of Industrial Chemistry*; Gerhartz, W., Ed.; VCH Verlagsgesellschaft mbH: Weinheim, 1985; Vol. A2, pp. 57-97.

6. Lamothe, P. J.; McCormick, P. G. *Anal. Chem.* **1973**, *45*, 1906.

7. Madder, A.; Farcy, N.; Hosten, N. G. C.; De Muynck, H.; De Clerq, P. J.; Barry, J.; Davis, A. P. *Eur. J. Org. Chem.* **1999**, 2787.

8. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 95.

9. Shah, A.; Rahman, S. S.; de Biasi, V.; Camilleri, P. *Anal. Commun.* **1997**, *34*, 325.

10. Vojkovsky, T. *Pept. Res.* **1995**, *8*, 236.

11. Chu, S. S.; Reich, S. H. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1053.

12. Nabbs, B. K., Ph.D. thesis, University of Canterbury, 1999.

13. Abell, A. D.; Martyn, D. C.; May, B. C. H.; Nabbs, B. K. *Tetrahedron Lett.* **2002**, *43*, 3673.

14. (a) Blockhuys, F.; Hinchley, S. L.; Robertson, H. E.; Blake, A. J.; McNab, H.; Despinoy, X. L. M.; Harris, S. G.; Rankin, D. W. H. *J. Chem. Soc., Perkin Trans. 2* **2001**, 2195. (b) Blake, A. J.; McNab, H.; Morrison, R. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2145.

15. Chakrabati, P.; Dunitz, J. D. *Helv. Chim. Acta* **1982**, *65*, 1555.

16. Nygaard, L.; Nielsen, J. T.; Kirchheiner, J.; Maltesen, G.; Rastrup-Andersen, J.; Sorensen, G. O. *J. Mol. Struc.* **1969**, *3*, 491.

17. Solomons, T. W. G. In *Organic Chemistry*; 4th ed.; John Wiley & Sons, Inc.: New York, 1988, pp. 566-647.

18. McLafferty, F. W.; Turecek, F. *Interpretation of Mass Spectra*; 4th ed.; University Science Books: Mill Valley, 1993.

19. Johnstone, R. A. W.; Rose, M. E. In *Chemistry and Biochemistry of the Amino Acids*; Barrett, G. C., Ed.; Chapman and Hall Ltd: London, 1985, pp. 480-524.

20. Leimer, K. R.; Rice, R. H.; Gehrke, C. W. *J. Chromatogr.* **1977**, *141*, 121.

21. Leimer, K. R.; Rice, R. H.; Gehrke, C. W. *J. Chromatogr.* **1977**, *141*, 355.

22. Fairwell, T.; Brewer, Jr., H. B. *Anal. Biochem.* **1980**, *107*, 140.

CHAPTER FIVE

DEVELOPMENT OF 5-(HYDROXYMETHYL)PYRROLE-BASED DNA ALKYLATING AGENTS

5.1 INTRODUCTION

Cancer is a disease characterised by the uncontrolled growth and division of cells within the body.[1] Treatment of a patient suffering from cancer is often dependent on the administration of therapeutic agents which inhibit the growth of tumour cells, resulting in destruction of the tumour by the body's immune system. One class of antitumour agent relies on alkylation of the genetic material for activity, whereby a covalent bond is formed between the compound and a nucleophilic site within the DNA. A wide range of reactive functionalities can alkylate DNA, and in particular the nitrogen mustards such as chlorambucil (**1.51**, Figure 5.1) have become the most common alkylating agents.[2] DNA alkylation has also been effected by hydroxymethylpyrrole-based compounds. The bis-alcohols **5.1**,[3] **5.2**[4] and **1.69**[5] were determined to alkylate DNA. Greater affinity for the genetic material was endowed upon **5.2** and **1.69** by the attachment of minor groove binding and intercalating moieties respectively.

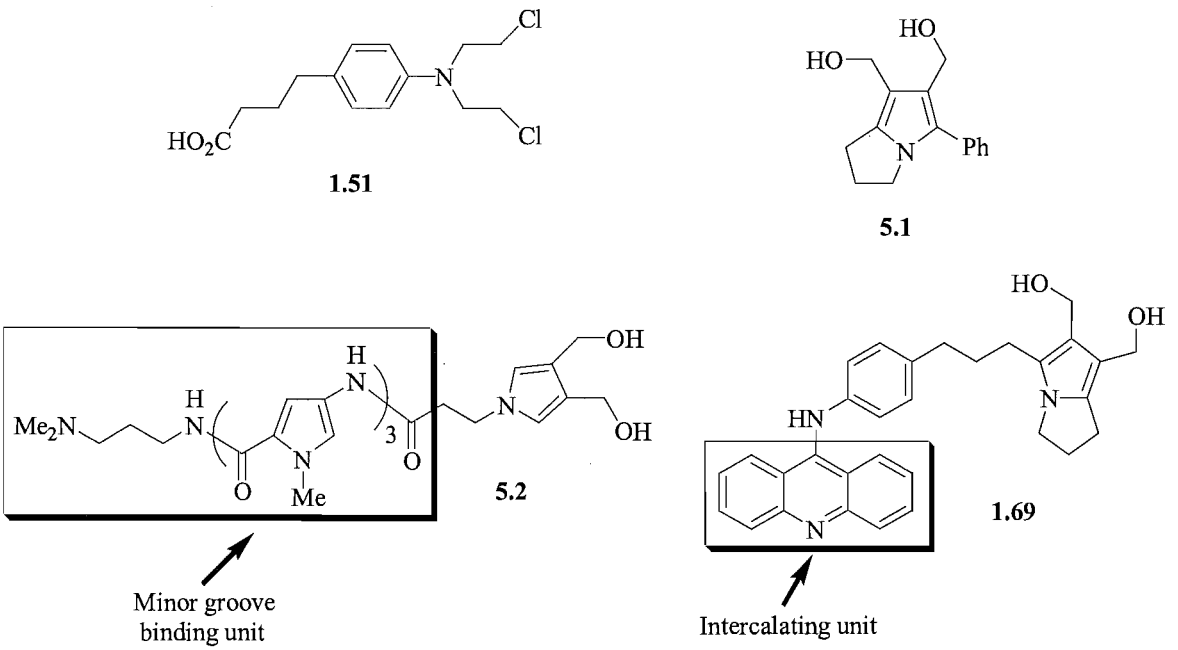
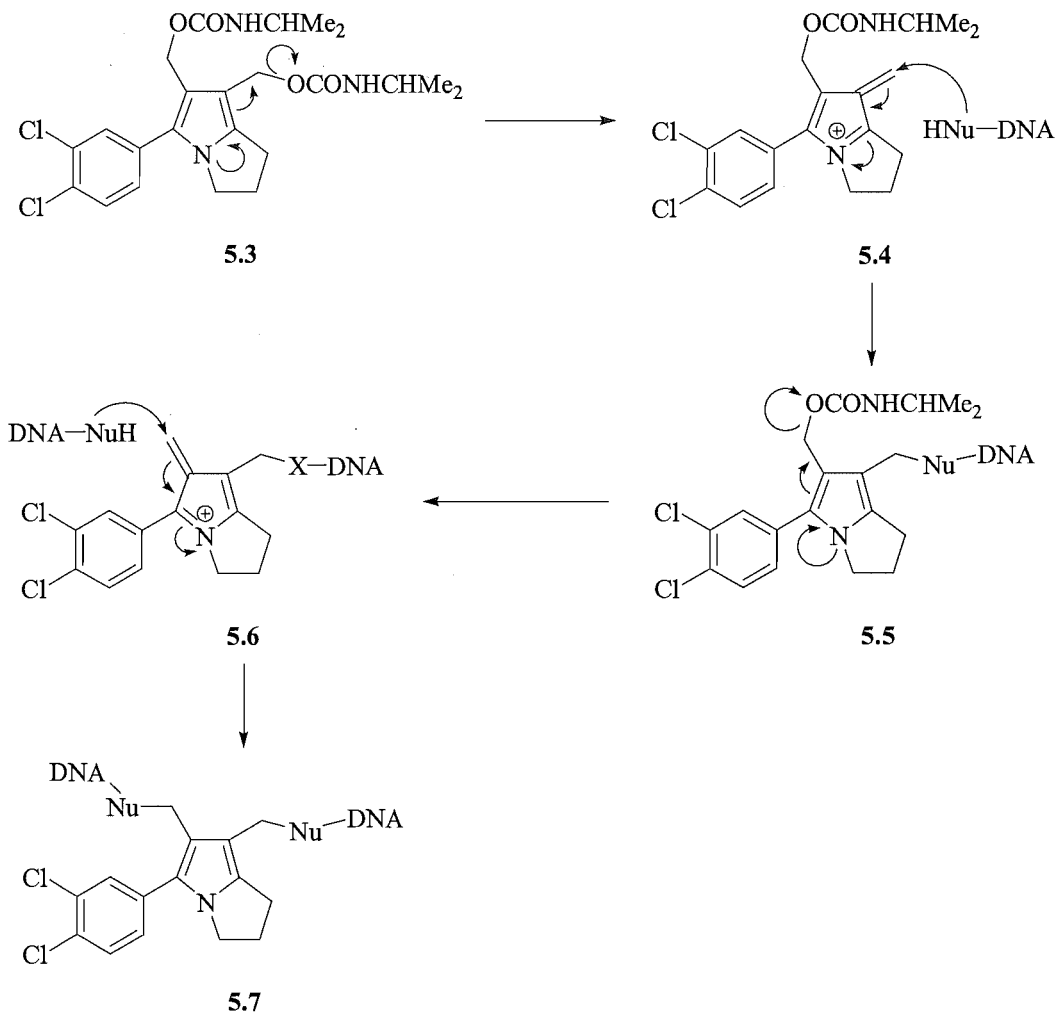


Figure 5.1. Structure of the representative nitrogen mustard chlorambucil (**1.51**), and the hydroxymethylpyrrole-based DNA alkylators **5.1**, **5.2** and **1.69**.

A mechanism for DNA alkylation by pyrrole-derived cross-linking agents has been proposed[6] (Scheme 5.1). The pyrrolizine **5.3**, which was reported to cross-link short oligonucleotides,[7] is converted to the reactive azafulvene **5.4**, which is subsequently attacked by a DNA nucleophile to give adduct **5.5**. This reaction sequence occurs again to give the cross-linked adduct **5.7**.



Scheme 5.1. Cross-linking of DNA by **5.3**. The nucleophilic sites in DNA are represented as DNA-NuH.

Other pyrrolic compounds display potential as antitumour agents. The moderate anticancer cell line activity of twelve 5-(hydroxymethyl)pyrrole-based metabolites, isolated from the Atlantic sponge *Mycale micracanthoxea*, was reported by Ortega, *et al.*[8] The representative examples **5.8a** (mycalazol 5) and **5.8b** (mycalazol 11) are displayed in

Figure 5.2. Compounds of type **5.8** possess an acyl side chain, of variable length and unsaturation, at the C2 position. A previous report[9] from these laboratories detailed a synthetic route that afforded this class of compounds by reaction of a stannylpyrrole and fatty acid chlorides under Stille coupling conditions. While compounds of type **5.8** displayed moderate activity against the P388 murine leukaemia cell line, this study did illustrate the potential application of the 5-(hydroxymethyl)pyrrole moiety toward the development of compounds with anticancer activity.

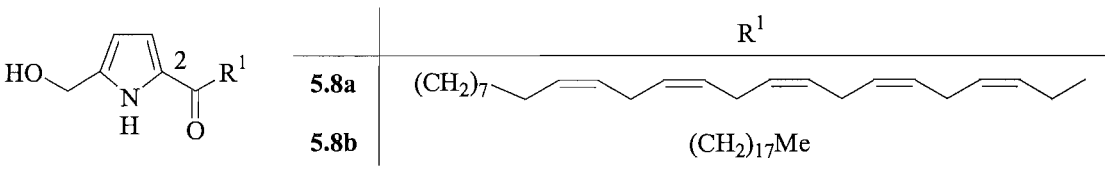


Figure 5.2. Structure of mycalazol 5 (**5.8a**) and mycalazol 11 (**5.8b**).

The 5-(hydroxymethyl)pyrrole functionality was utilised in Chapter Three as a reactive moiety to which an enzyme active site residue becomes covalently attached. Concurrent to that study was the development of 5-(hydroxymethyl)pyrroles, based on the general structure **5.9** (Figure 5.3), as DNA alkylating agents. Whilst the 5-(hydroxymethyl)pyrrole moiety possessed by compounds of type **5.8** is retained in the structure of **5.9**, the aliphatic chain of **5.8** has been replaced by an intercalating moiety, appended to C2 via an alkyl linker.

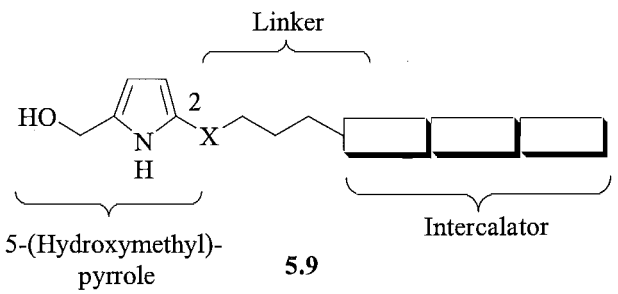


Figure 5.3. General structure **5.9** of the proposed DNA alkylating agents.

Compounds of type **5.9** possess three important structural elements. The first is an intercalator, a planar aromatic moiety that is able to insert between pairs of complementary

bases in the DNA double helix (see Chapter One, Section 1.3 for further discussion). This would introduce the property of DNA affinity into compounds of type **5.9**. Figure 5.4 illustrates the proposed interaction of **5.9** with the genetic material, with the intercalator inserted between the base pairs. The second element is an alkyl linker, which endows flexibility upon **5.9,** allowing positioning of the 5-(hydroxymethyl)pyrrole moiety into close proximity to DNA nucleophiles. The activity displayed by compounds of type **5.9** towards cancer cells was proposed to be dependent on the alkylation of DNA by the 5-(hydroxymethyl)pyrrole functionality.

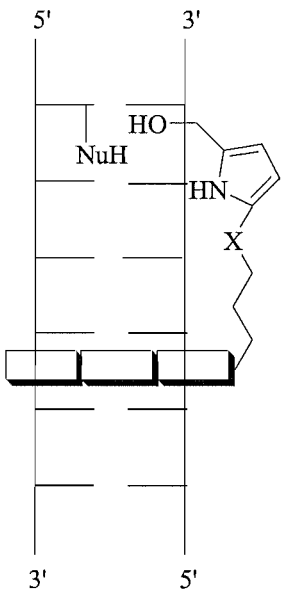
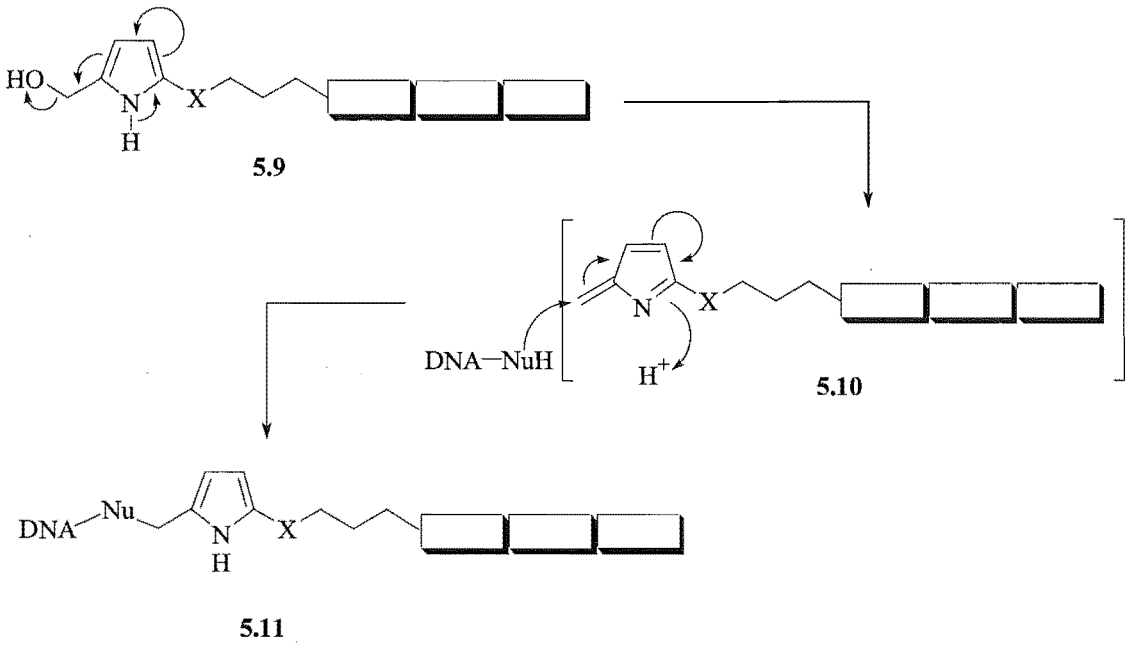


Figure 5.4. Schematic representation of the interaction of **5.9** with DNA (NuH = reactive site on DNA). The double helix is represented in an unwound state for clarity.

DNA alkylation by compounds of type **5.9** is proposed to occur by the mechanism illustrated in Scheme 5.2. This process is analogous to those postulated for the 5-(hydroxymethyl)pyrrole-based α-chymotrypsin inhibitors (see Chapter Three, Section 3.1), and the pyrrole-derived cross-linking agents (see Scheme 5.1). To this end, azafulvene **5.10** is generated from the 5-(hydroxymethyl)pyrrole **5.9**, and subsequent attack by a DNA nucleophile affords adduct **5.11**.

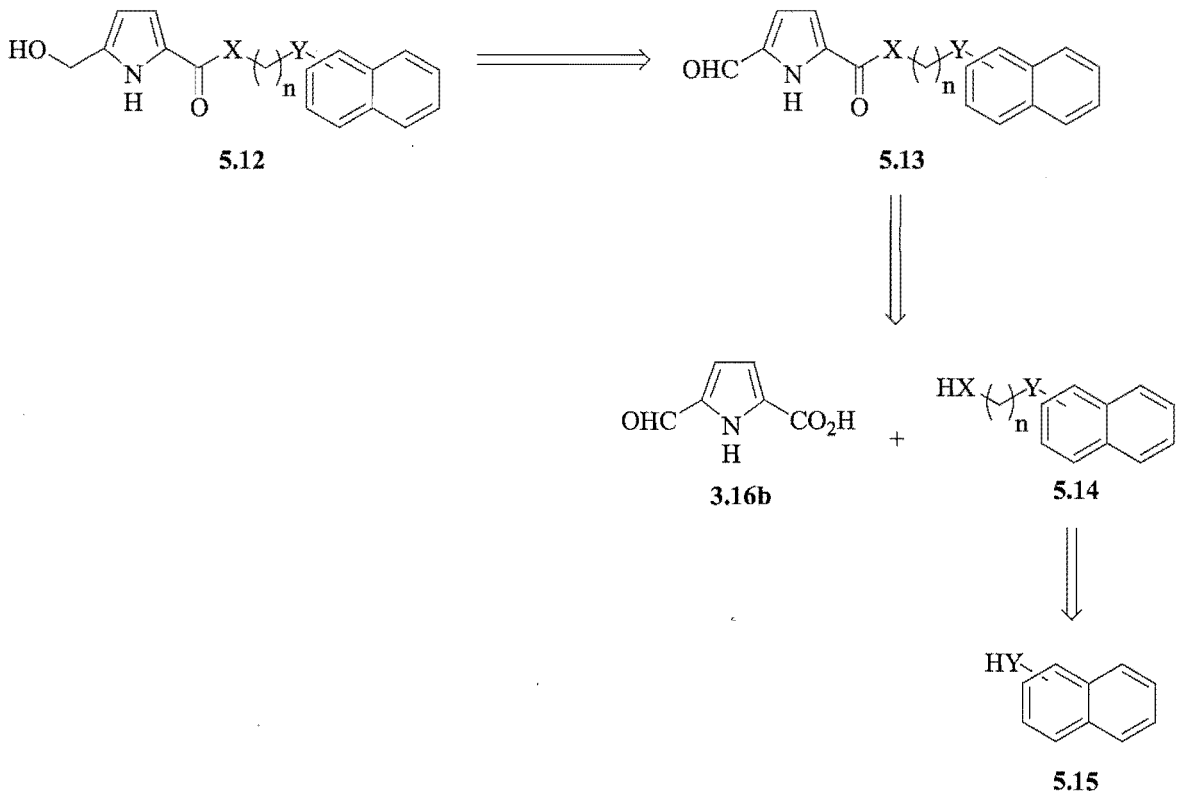


Scheme 5.2. Proposed reaction of **5.9** with a nucleophile present within DNA (represented as DNA-NuH) to afford adduct **5.11**.

This chapter initially describes the synthesis towards compounds of type **5.9**, with structural variations restricted to the intercalating moiety. The prepared compounds were then assayed for activity against the P388 cancer cell line to give a preliminary understanding of the structure-activity relationships for this class of compound. Additionally, compounds of type **5.9** were assayed against representative microbes and viruses to determine the activity of this class of compounds against these important pharmaceutical targets.

5.2 MODEL SYNTHETIC STUDIES TOWARDS 5-(HYDROXYMETHYL)-PYRROLE-BASED DNA ALKYLATORS

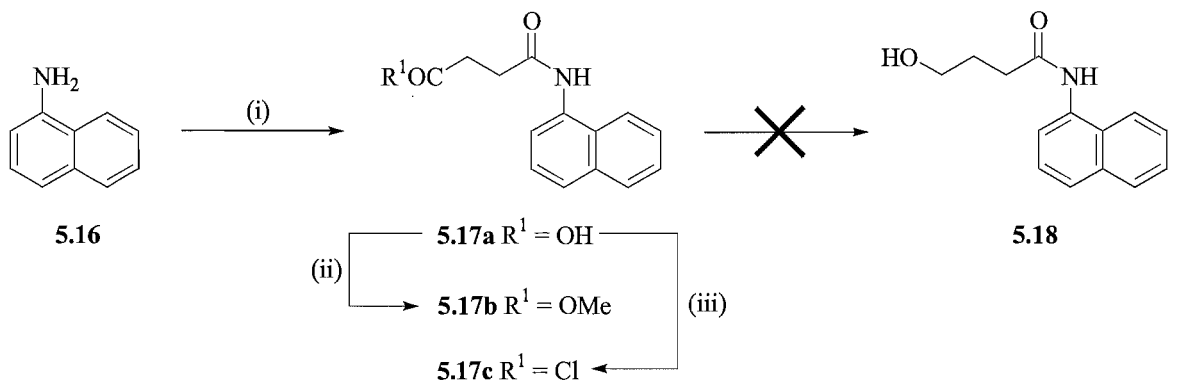
A model synthetic study, with the general structure **5.12** (Scheme 5.3) the initial target, was undertaken to determine the optimal route to compounds of type **5.9**. A naphthyl moiety was chosen to act as an intercalator surrogate due to the availability of suitable naphthalene derivatives. The retrosynthetic analysis outlined in Scheme 5.3 illustrates that compounds of type **5.12** would be best obtained by reduction of **5.13**. Disconnection of **5.13** gives the pyrrole acid **3.16b** and **5.14**, which is the linker-carbocycle adduct derived from a naphthyl starting material (**5.15**).



Scheme 5.3. Retrosynthetic analysis for **5.12**.

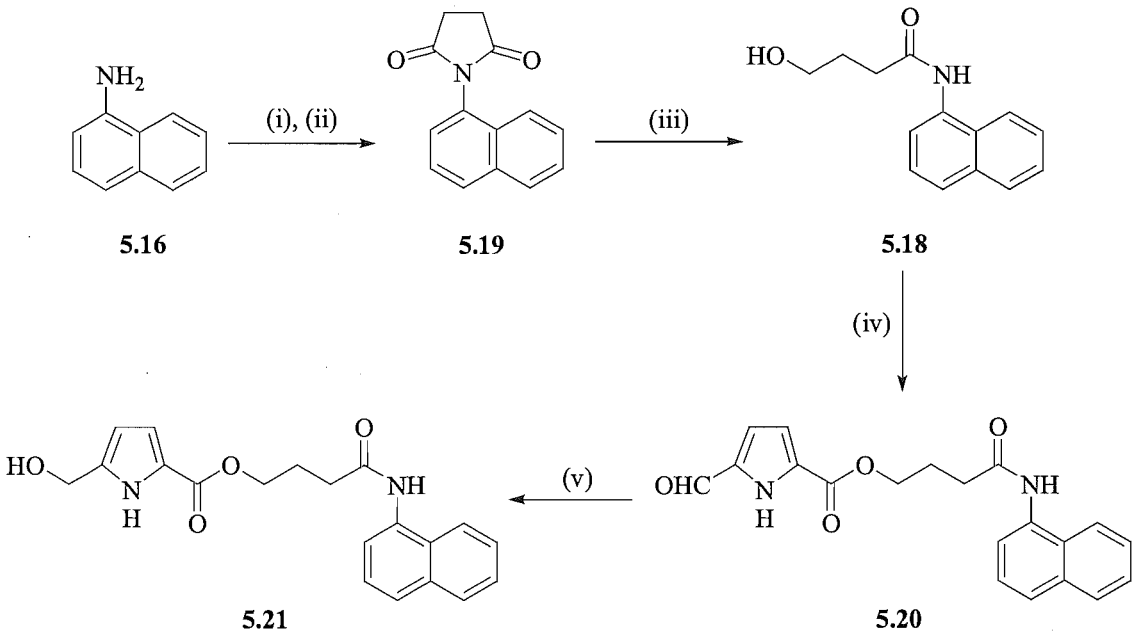
Preparation of the key intermediate represented by **5.14** was initially undertaken. This target required a nucleophilic moiety for subsequent coupling to **3.16a** (see Scheme 5.3). The aromatic compound chosen to act as an intercalator surrogate was naphthalen-1-

ylamine (**5.16**, Scheme 5.4). Reaction of **5.16** with succinic anhydride in refluxing toluene[10] gave the acid **5.17a** in 91% yield. Attempted reduction of **5.17a** to the alcohol **5.18** with LAH gave a complex mixture of products by ^{1}H NMR spectroscopy and TLC. Derivatisation of the carboxylic acid of **5.17a** was considered a means to generate a moiety more suited for reduction. To this end, esterification of **5.17a** with diazomethane afforded **5.17b** in 70% yield. However, reduction of **5.17b** with LAH, or the milder reducing agent DIBALH, again gave a mixture of products by ^{1}H NMR spectroscopy and TLC. Alternatively, **5.17a** was converted to its acid chloride **5.17c** by reaction with oxalyl chloride/DMF. Reduction of **5.17c** with either LAH or DIBALH gave a mixture of products by ^{1}H NMR spectroscopy. At this stage, work towards the reduction of **5.17a-c** to the alcohol **5.18** was abandoned.



Scheme 5.4. *Reagents and conditions:* (i) Succinic anhydride, PhMe, reflux, 15 min (91%). (ii) CH_2N_2, THF, r.t., 15 h (70%). (iii) $(COCl)_2$, $DMF_{(cat.)}$, CH_2Cl_2, r.t., 1 h (not isolated).

Preparation of the key intermediate **5.18** was achieved as illustrated in Scheme 5.5. Amine **5.16** was reacted with succinic anhydride in refluxing toluene, then treated with sodium acetate in acetic anhydride[11] to afford the succinimide **5.19** in 86% yield over two steps. Reduction and ring-opening of **5.19** with sodium borohydride in 6:1 *iso*-propanol/water[12] gave **5.18** in 71% yield. A BOP-Cl-mediated coupling[13] of **3.16b** and **5.18** afforded **5.20**, and reduction of the coupled product with lithium borohydride gave **5.21** in 18% yield over two steps.



Scheme 5.5. *Reagents and conditions:* (i) Succinic anhydride, PhMe, reflux, 15 min. (ii) CH_3CO_2Na, Ac_2O, 70-80°C, 4.5 h (86% over two steps). (iii) $NaBH_4$, 6:1 $^iPrOH/H_2O$, r.t., 17 h (71%). (iv) **3.16b**, BOP-Cl, Et_3N, CH_2Cl_2, r.t., 64 h. (v) $LiBH_4$, THF, -78°C, 1 h, then r.t., 1 h (18% over two steps).

5.3 ATTEMPTED SYNTHESIS OF A 5-(HYDROXYMETHYL)PYRROLE-BASED DNA ALKYLATOR WITH AN ANTHRACENE INTERCALATIVE MOIETY

Preparation of the naphthyl-based compound **5.21** yielded a general synthetic method for the introduction of a range of aromatic moieties into compounds of type **5.9**. Hence, the structure-activity relationships for this class of compounds could be investigated. Generally, a fused tricyclic structure is required for intercalation[14] (although some exceptions exist, see Chapter One, Section 1.3 for further discussion). Therefore, an anthracene ring system was chosen to be the first example of a "true" intercalator incorporated into compounds of type **5.9**. The synthetic target **5.22** is illustrated in Figure 5.5, in addition to bisantrene (**5.23**), an example of a clinically trialed anticancer agent based on an anthracene ring system.[15]

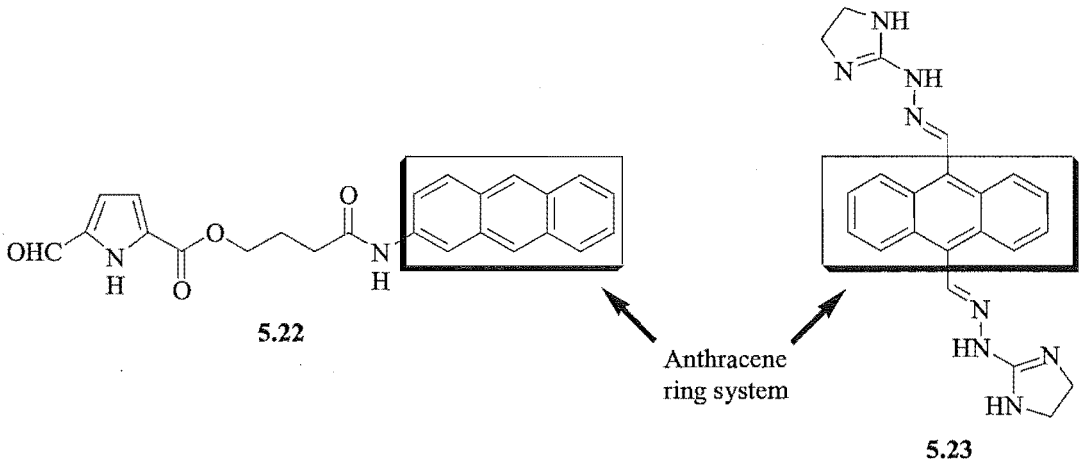
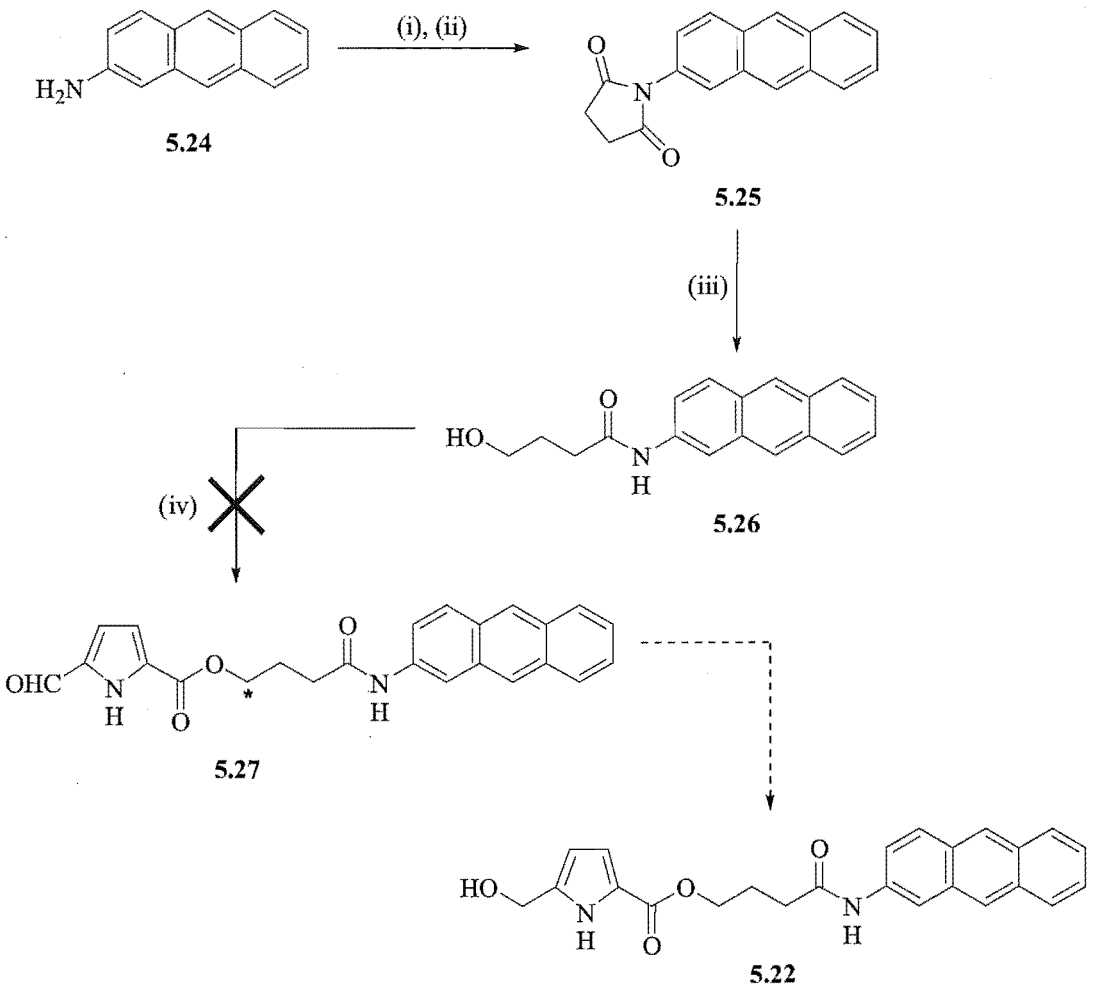


Figure 5.5. Structures of the synthetic target **5.22** and bisantrene (**5.23**). The anthracene ring system is highlighted in both compounds.

The synthesis of **5.22** (Scheme 5.6) was attempted in a manner analogous to that of **5.21**. Anthracen-2-ylamine (**5.24**) was reacted with succinic anhydride, followed by treatment with sodium acetate/acetic anhydride, to afford the succinimide **5.25** in 89% yield over two steps. Reduction/ring opening of **5.25** with sodium borohydride gave alcohol **5.26** with a quantitative return of material, and this crude product was coupled to the pyrrole acid **3.16a** by BOP-Cl methodology. However, the ^{1}H NMR spectrum obtained after workup revealed a weak resonance at δ 4.28 ppm, corresponding to $CO_2C\underline{H}_2$ (marked * in Scheme 5.6), hence **5.27** was a minor product. The low yield of coupled product resulted from the insolubility of **5.26** in dichloromethane. Prior studies revealed a polar solvent such as DMF did not effect the coupling of **3.16a** to **5.18a** to afford **5.20** (see Scheme 5.5), and as such further attempts towards the synthesis of **5.22** were abandoned.



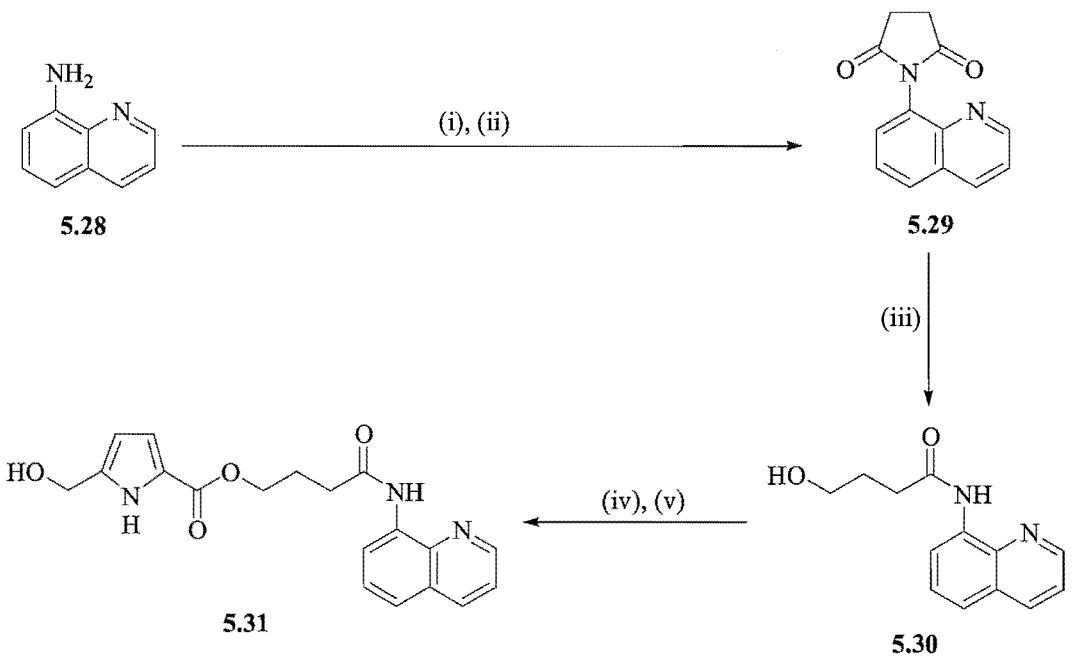
Scheme 5.6. *Reagents and conditions:* (i) Succinic anhydride, PhMe, reflux, 1 h. (ii) CH_3CO_2Na, Ac_2O, 70-80°C, 4.5 h (89% over two steps). (iii) $NaBH_4$, 6:1 $^{i}PrOH/H_2O$, r.t., 17 h (quantitative crude yield). (iv) **3.16b**, BOP-Cl, Et_3N, CH_2Cl_2, r.t., 64 h.

5.4 ATTEMPTED SYNTHESIS OF A 5-(HYDROXYMETHYL)PYRROLE-BASED DNA ALKYLATOR WITH A HETEROCYCLIC INTERCALATOR

A heterocyclic intercalator, in place of the carbocyclic moiety possessed by **5.26**, was proposed to overcome the low yield encountered in the coupling step between **3.16a** to **5.26** due to an improved solubility profile. The following section details the work towards the synthesis of a compound of type **5.9** that possessed such a moiety.

5.4.1 Model synthetic studies with 8-aminoquinolene as an intercalator surrogate

Quinolin-8-ylamine (**5.28**, Scheme 5.11) was used as a model heterocycle to determine whether the synthetic route developed for the naphthyl-based analogue **5.21** was applicable to an example with a heterocyclic moiety. To this end, **5.28** was reacted with succinic an-



Scheme 5.7. *Reagents and conditions:* (i) Succinic anhydride, PhMe, reflux, 15 min. (ii) CH_3CO_2Na, Ac_2O, 70-80°C, 4.5 h (79% over two steps). (iii) $NaBH_4$, 6:1 $^iPrOH/H_2O$, r.t., 17 h. (iv) **3.16b**, BOP-Cl, Et_3N, CH_2Cl_2, r.t., 64 h. (v) $LiBH_4$, THF, -78°C, 1 h, then r.t., 1 h (11% over three steps).

hydride, then treated with sodium acetate/acetic anhydride, to afford the succinimide **5.29** in 79% yield over two steps. Reduction of **5.29** with sodium borohydride gave the alcohol **5.30**, which was subsequently coupled to **3.16b** by BOP-Cl methodology. The coupled product was reduced with lithium borohydride to give the desired product **5.31** in 11% yield over three steps.

5.4.2 Attempted synthesis of a 5-(hydroxymethyl)pyrrole-based DNA alkylator with an acridine intercalative moiety

Preparation of the putative intercalating alkylator **5.32** (Figure 5.6), which incorporated an acridine ring system into its structure, was subsequently undertaken. Many antitumour agents intercalate as a result of an acridine group, and the example of amsacrine (**1.57**)[16] is displayed in Figure 5.6 (see Chapter One, Section 1.3 for further examples of compounds with acridine moieties).

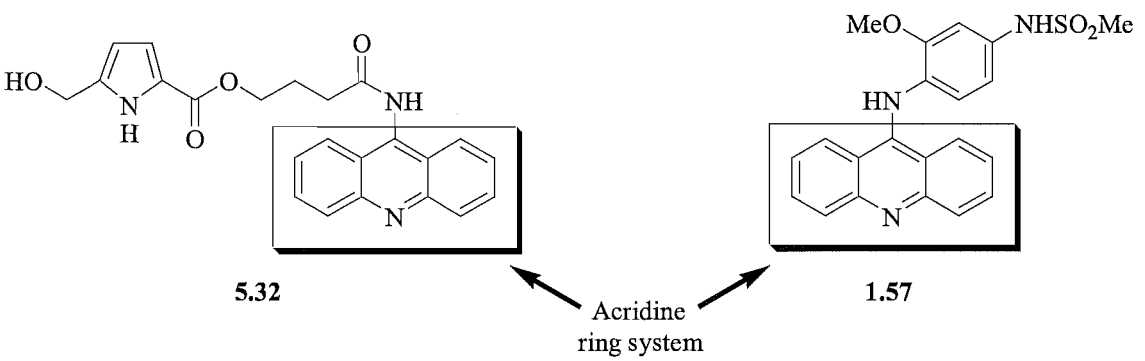
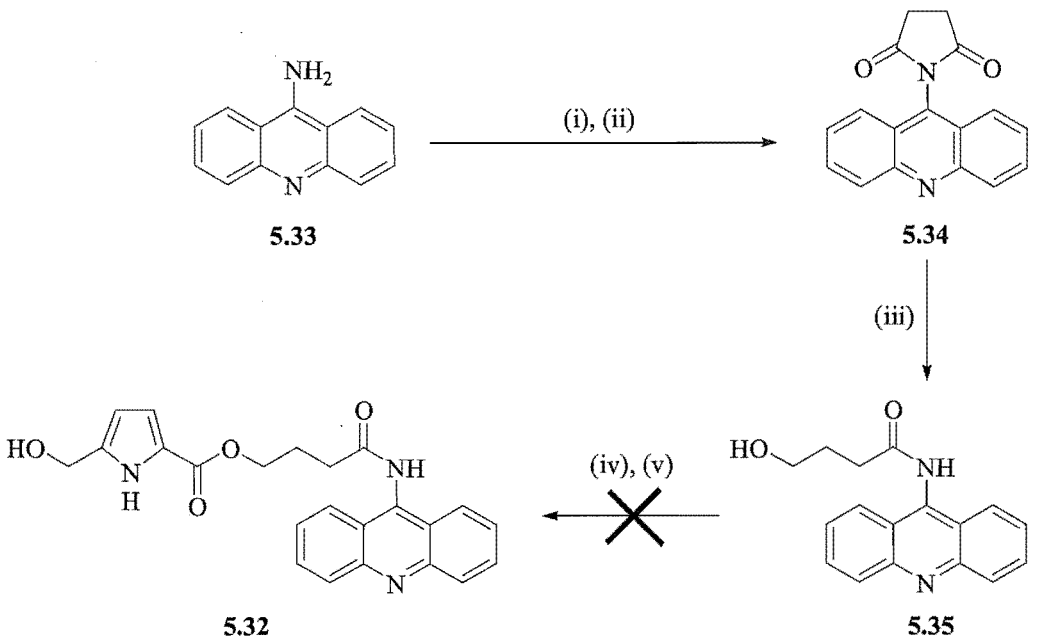


Figure 5.6. Structures of the synthetic target **5.32** and amsacrine (**1.57**). The acridine ring system is highlighted in both compounds.

The synthetic route to **5.32** is illustrated in Scheme 5.8. Acridin-9-ylamine (**5.33**) was treated with succinic anhydride in refluxing DMF in place of toluene, due to the insolubility of **5.33** in non-polar solvents. Treatment of the intermediate acid with sodium acetate/acetic anhydride afforded the succinimide **5.34** in 35% yield over two steps. Reduction and ring opening of the succinimide gave the alcohol **5.35** in 89% crude yield, which was subsequently reacted with pyrrole acid **3.16b** under BOP-Cl coupling

conditions. The coupled product was reduced with lithium borohydride to afford **5.32** in a very low yield (<5%) and an impure state despite several attempts to effect purification by chromatography and recrystallisation. HRMS analysis confirmed the presence of **5.32** by the accurate mass obtained for protonated product ($C_{23}H_{22}N_3O_4$ = 404.1600 g mol^{-1}, calculated mass = 404.1610 g mol^{-1}).



Scheme 5.8. *Reagents and conditions:* (i) Succinic anhydride, DMF, reflux, 30 min. (ii) CH_3CO_2Na, Ac_2O, 70-80°C, 4.5 h (35% over two steps). (iii) $NaBH_4$, 6:1 $^{i}PrOH/H_2O$, r.t., 17 h (89% crude). (iv) **3.16b**, BOP-Cl, Et_3N, CH_2Cl_2, r.t., 64 h. (v) $LiBH_4$, THF, -78°C, 1 h, then r.t., 1 h.

The mixture containing **5.32** was analysed by reverse-phase HPLC (Figure 5.7). Expansion of the trace between 7.9-10.7 min revealed four compounds of similar polarity. Other peaks in the spectrum are solvent and impurity peaks found in all HPLC traces that utilise this particular solvent system (see Experimental for details). The mass spectrum obtained from analysis of the mixture by electrospray ionisation LRMS contained ions corresponding to protonated **5.32** ($C_{23}H_{22}N_3O_4$ = 404.2 g mol^{-1}) and protonated 5-formylpyrrole precursor ($C_{23}H_{20}N_3O_4$ = 402.2 g mol^{-1}). Ions were also observed that were two mass units lower than that expected for **5.35**, and one mass unit lower than that expected for **3.16b**, which suggested that these compounds were present, and

rearrangements were occurring during mass spectrometry to give the observed ions. Because of the low yields and extreme difficulties encountered with purification, the synthesis of **5.32** was abandoned.

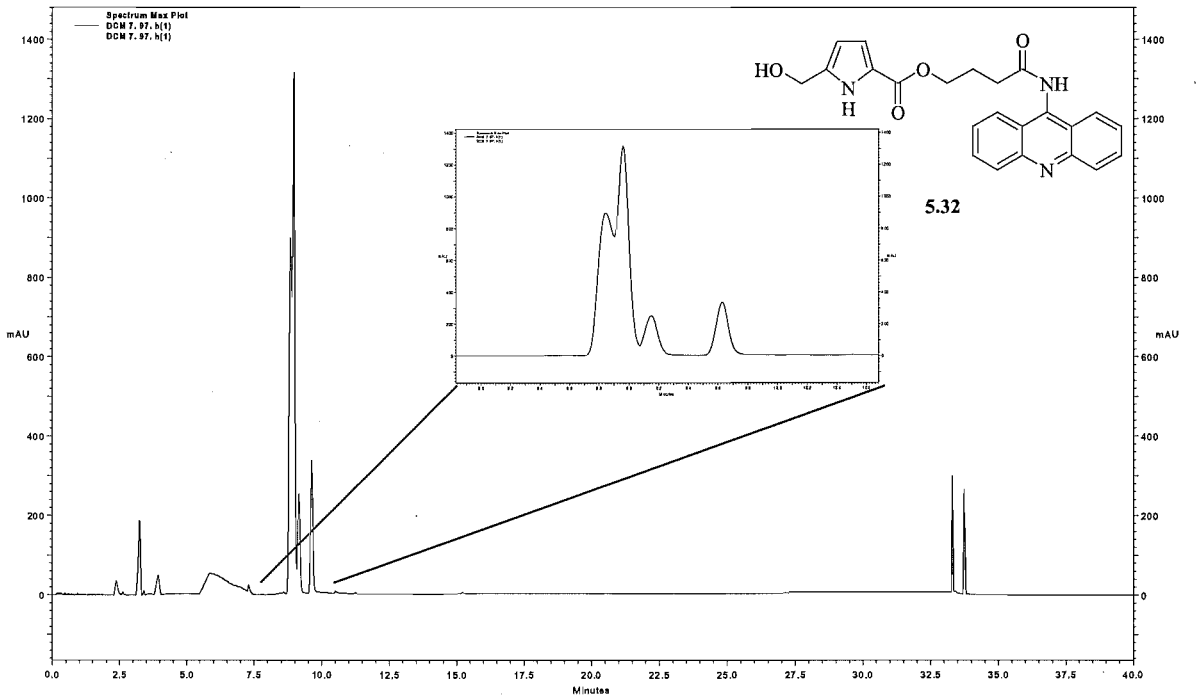


Figure 5.7. HPLC trace of **5.32**, with the region between 7.9-10.7 min expanded to illustrate the impure nature of the sample.

5.5 ATTEMPTED SYNTHESIS OF A 5-(HYDROXYMETHYL)PYRROLE-BASED DNA ALKYLATOR CAPABLE OF ACTING AS A THREADING INTERCALATOR

Threading intercalation is a binding mode whereby an intercalative moiety directs two substituents into each groove of the double helix simultaneously[17] (see Chapter 1, Section 1.3.2 for further discussion). The synthetic target **5.36** (Figure 5.8) was proposed to interact with DNA in this manner. Two distal 5-(hydroxymethyl)pyrrole-linker groups are appended to an acridine core, *i.e.* the reactive functionalities could potentially alkylate DNA at two separate sites per molecule of **5.36**. Figure 5.8 also illustrates the representative threading intercalator **1.78**,[17] and the structural similarities between **5.36** and **1.78** are highlighted.

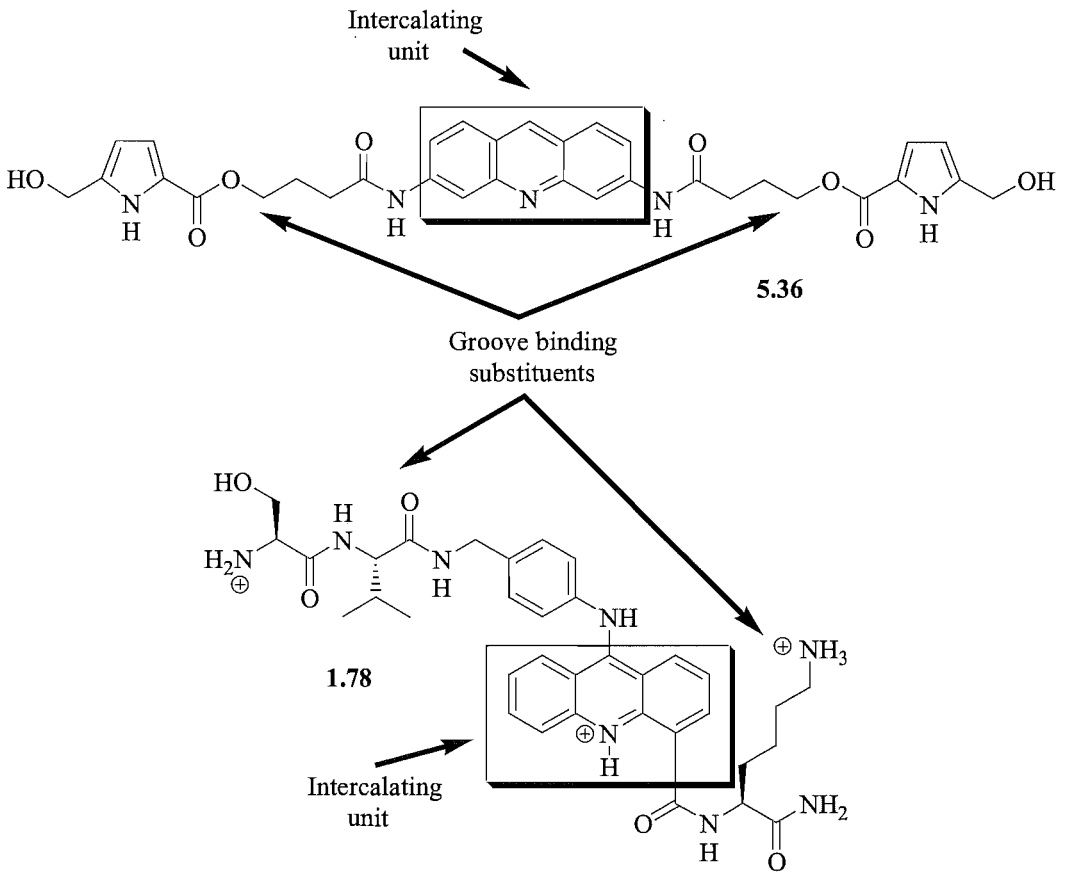
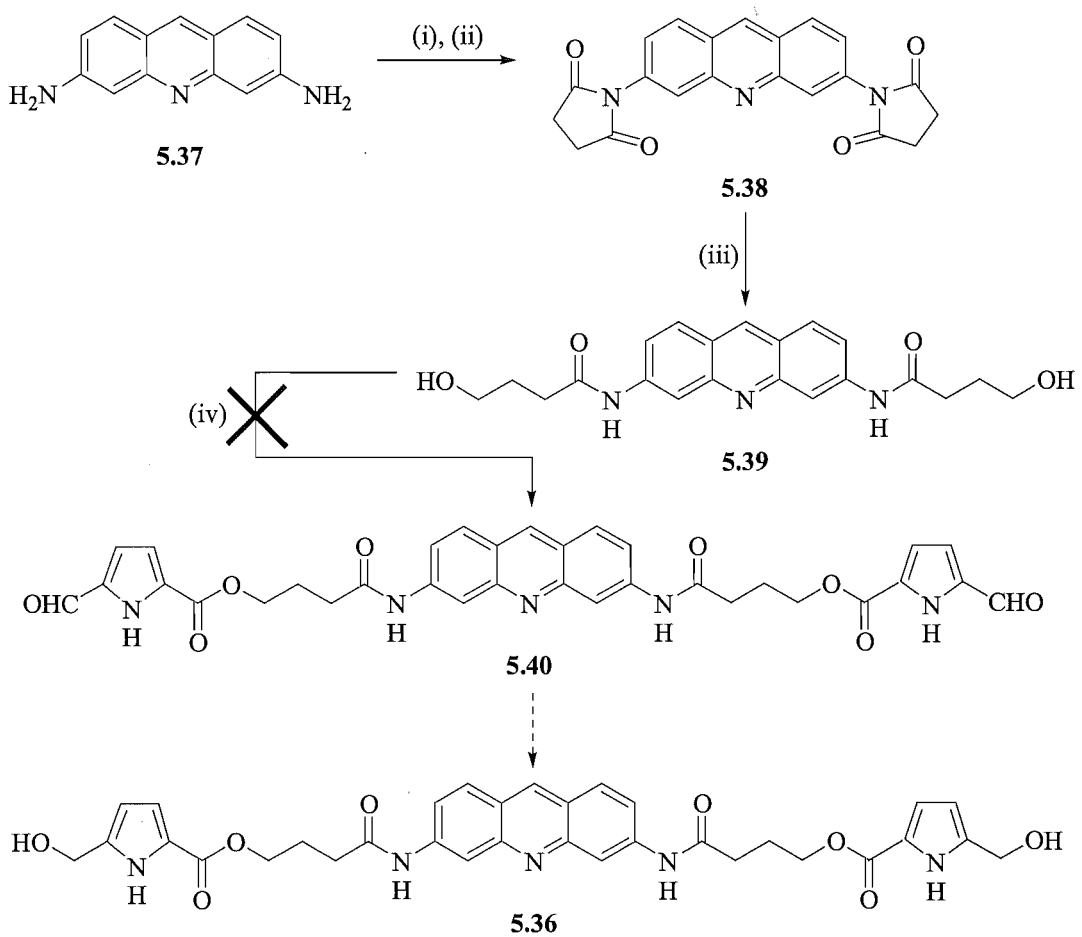


Figure 5.8. Synthetic target **5.36**, and the representative threading intercalator **1.78**. The acridine and groove binding structural features are highlighted in both compounds.

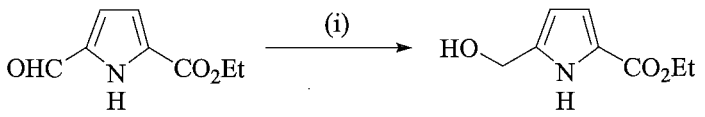
To this end, 3,6-diaminoacridine (**5.37**, Scheme 5.9) was reacted with succinic anhydride in refluxing pyridine,[18] then treated with sodium acetate/acetic anhydride, to afford the succinimide **5.38** in a low yield of 5% over two steps. Reduction of **5.38** with sodium borohydride gave crude alcohol **5.39** (determined by ^{1}H NMR spectroscopy) with a mass return of 84%. The attempted BOP-Cl-mediated coupling of **3.16b** to **5.39** gave an intractable mixture. Analysis by electrospray ionisation LRMS revealed an ion at 624.0 g mol^{-1}, corresponding to the mass of protonated **5.40**, however the ^{1}H NMR spectrum of the mixture indicated that **5.40** was a minor product. The synthesis of **5.36** was abandoned at this stage due to the low yields encountered in the preparation of key intermediates (*cf* synthesis of **5.22** and **5.32**).



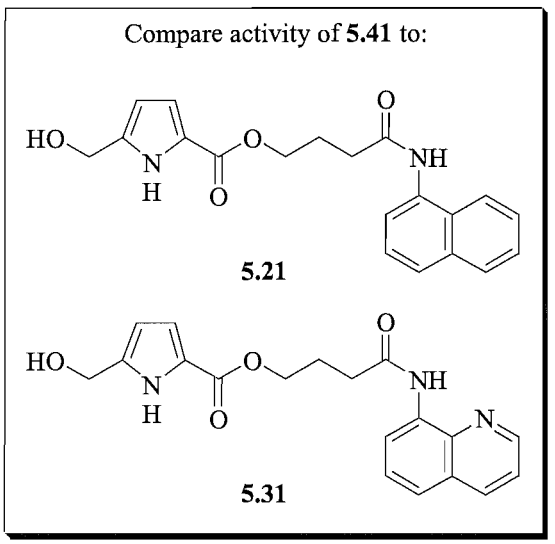
Scheme 5.9. *Reagents and conditions:* (i) Succinic anhydride, pyridine, reflux, 2 h. (ii) CH_3CO_2Na, Ac_2O, 70-80°C, 4.5 h (5% over two steps). (iii) $NaBH_4$, 6:1 ^{i}PrOH/H_2O, r.t., 17 h (84% crude). (iv) **3.16b**, BOP-Cl, Et_3N, CH_2Cl_2, r.t., 64 h.

5.6 SYNTHESIS OF A 5-(HYDROXYMETHYL)PYRROLE SUBSTRUCTURE ANALOGUE

The postulated mechanism of DNA alkylation by compounds of type **5.9** in Scheme 5.2 is mediated by the latent reactivity of the 5-(hydroxymethyl)pyrrole functionality. The biological activity of the 5-(hydroxymethyl)pyrrole substructure analogue **5.41** was to be compared against that elicited by **5.21** and **5.31** to determine the importance of this substructure for activity. Any additional activity displayed by **5.21** and **5.31** would result from interaction of the linker-aromatic structure possessed by these compounds with the genetic material. To this end, the 5-formylpyrrole **3.16a** (Scheme 5.10) was reduced with lithium borohydride to give **5.41**, in 76% yield, for use in the assays.



3.16a **5.41**



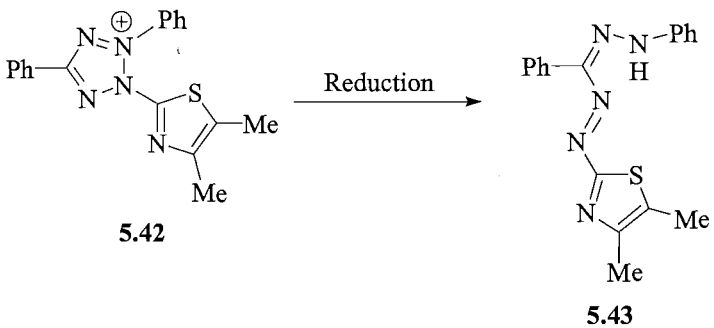
Scheme 5.10. *Reagents and conditions:* (i) $LiBH_4$, THF, -78°C, 1 h, then r.t., 1 h (76%).

5.7 BIOLOGICAL ASSAYS

The following subsections give background information to the antitumour, antiviral and antimicrobial assays, in addition to the activities of six compounds (see Table 5.1) in each assay. Naphthylen-1-ylamine (**5.16**) and quinolin-2-ylamine (**5.28**) were submitted for comparison of their activities to that of the 5-(hydroxymethyl)pyrrole-based compounds that possessed these ring systems (**5.21** and **5.31** respectively). A representative tricyclic aromatic starting material (**5.37**) was submitted for comparison of its biological activity to that of the bicyclic starting materials **5.16** and **5.28**. Submission of the 5-(hydroxymethyl)pyrrole substructure analogue **5.41** allowed elucidation of this moieties biological activity. Additionally, the binding constant of **5.31** to DNA was determined.

5.7.1 Antitumour assay

The antitumour assay is the most sensitive assay for cytotoxicity. Results from the P388 assay are given as an ID_{50} value, which expresses the sample concentration required to reduce the growth of P388 murine leukaemia cells by 50%, in comparison to the control cells.[19] The ID_{50} value is determined using the absorbance values (detected at 540 nm) obtained when the yellow dye MTT tetrazolium (**5.42**, Scheme 5.11) is reduced in the mitochondria of viable leukaemia cells to the purple coloured MTT formazan (**5.43**).

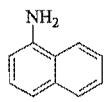
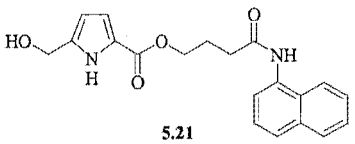
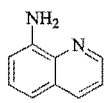
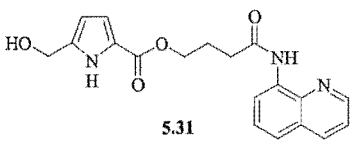
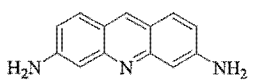
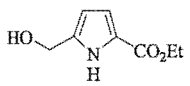


5.42 **5.43**

Scheme 5.11. Reduction of yellow MTT tetrazolium **5.42** to purple MTT formazan **5.43** by viable leukaemia cells.

Increased cytotoxicity is revealed by a lower ID_{50} value. The ID_{50} value is expressed as μg mL^{-1}, and results that contain a > sign indicate that the sample was not concentrated enough for an ID_{50} value to be determined.

Table 5.1 displays the activity determined for **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41**. The standard method of expressing the ID_{50} values (column 4) has also been calculated to give ID_{50} values as molar concentrations (column 5). This was done to more accurately compare the activities of the compounds assayed against the P388 cancer cell line.

No.	Solvent system*	Sample concentration (mg mL^{-1})#	ID_{50} (μg mL^{-1})	ID_{50} (μM)
 **5.16**	A	10	87	611
 **5.21**	B	20	165	468
 **5.28**	A	10	13	88
 **5.31**	A	10	111	314
 **5.37**	B	1	2.0	9.4
 **5.41**	A	10	>125	>739

* A = MeOH, B = 3:1 MeOH/DMF
# Concentration of compound submitted for analysis

Table 5.1. Activity of **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41** against the P388 cell line.

A 23% increase in activity was observed for the 5-(hydroxymethyl)pyrrole **5.21** (ID_{50} = 468 μM), compared to its naphthyl starting material **5.16** (ID_{50} = 611 μM). In contrast, quinolin-8-amine **5.28** (ID_{50} = 88 μM) was 3.5-fold more active than the 5-(hydroxymethyl)pyrrole **5.31** (ID_{50} = 314 μM), derived in five steps from **5.28**. The 5-(hydroxymethyl)pyrrole substructure analogue **5.41** displayed no activity. All compounds were appreciably less active than **5.37**. The increased ability of **5.37** to restrict cell proliferation, compared to **5.16**, **5.21**, **5.28**, **5.31** and **5.41**, must result from the tricyclic ring system of **5.37** binding tightly to the genetic material by intercalation.

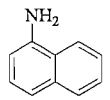
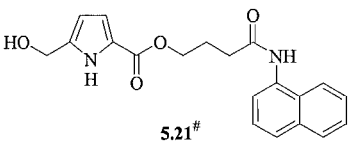
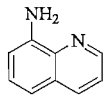
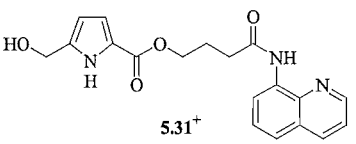
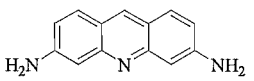
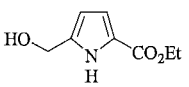
5.7.2 Antiviral assay

The membrane enveloped RNA virus Polio type 1 (Pfizer vaccine strain, PV 1) and the non-membrane enveloped DNA virus Herpes simplex type 1 (ATC VR 733, HSV 1) were the test viruses used in this assay. African green monkey kidney cells (BSC-1), grown on the surface of plastic wells, were used as host cells for the test viruses.

A scale is used to record the antiviral and cytotoxic zones of inhibition which surrounds the discs onto which the sample of interest was loaded:

ND	No discernable antiviral or cytotoxic effects
±	Minor effects located under the disc
+	Antiviral or cytotoxic zone 1-2 mm excess radius from the disc (25%)
2+	Antiviral or cytotoxic zone 2-4 mm excess radius from the disc (50%)
3+	Antiviral or cytotoxic zone 4-6 mm excess radius from the disc (75%)
4+	Antiviral or cytotoxic zone over the whole well (100%)
+*	Light toxicity throughout the whole well
?	Unable to determine level of antiviral activity due to strong cytotoxicity. Needs to be re-assayed at lower concentration.
?*	No antiviral activity as the virus is growing right up to the cytotoxic zone

Interpretation of the results can be difficult where both cytotoxic and antiviral effects are present because strong cytotoxicity will mask any antiviral activity within the cell. The results for **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41** are displayed in Table 5.2.

No.	PV 1		HSV 1	
	Antiviral effects	Cytotoxic effects	Antiviral effects	Cytotoxic effects
 **5.16**$^{+}$	?	4+	?	4+
 **5.21**$^{\#}$	ND	ND	ND	ND
 **5.28**$^{+}$	?*	2+	?*	2+
 **5.31**$^{+}$	?	4+	?*	3+
 **5.37**$^{+}$	?*	+	?*	2+
 **5.41**$^{+}$	ND	ND	ND	ND

$^{+}$ Activity tested in the solvent system and sample concentration specified in Table 5.1.
$^{\#}$ Activity tested in solvent system A at 10 mg mL^{-1}.

Table 5.2. Activity of **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41** against the Polio type 1 virus (PV 1) and Herpes simplex type 1 virus (HSV 1).

The antiviral activity of **5.16** could not be determined because of a strong cytotoxic effect (denoted as ?) towards the kidney cells used as viral hosts in the assay. Amines **5.28** and **5.37** displayed no antiviral effects beyond the cytotoxic region (denoted as ?*). The

antiviral activity of **5.31** against PV 1 could not be determined due to strong cytotoxicity. No antiviral effects were displayed by **5.31** against HSV 1 beyond the cytotoxic region. No antiviral or cytotoxic activity was observed for **5.21** and **5.41**.

5.7.3 Antimicrobial assay

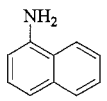
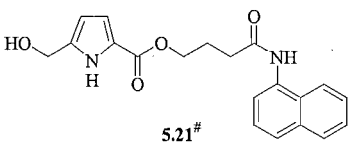
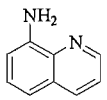
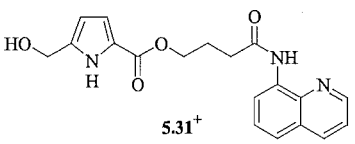
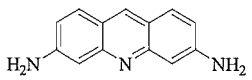
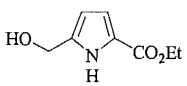
A zone of growth inhibition is used to test for antimicrobial activity. The samples of interest are adsorbed onto separate paper discs, allowed to dry and placed on a seeded agar plate of the organism of interest. The zone of growth inhibition around the disc is measured (mm) after an incubation period. Six test organisms were used in the assay (names in parentheses are the abbreviations used in Table 5.3):

Bacteria: *Escherichia coli* (*E coli*), *Bacillus subtilis* (*B sub*)*, *Pseudomonas aeruginosa* (*P aer*)

Fungi: *Candida albicans* (*C alb*), *Trichophyton mentagrophytes* (*T ment*)*, *Cladosporium resinae* (*C res*)

* = most sensitive organisms.

Table 5.3 presents the activity of **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41** against the six microbial species. The activity of these compounds is compared to that generated by discs containing 10 μg of gentamycin (an antibacterial agent selective for the Gram negative species *E. coli* and *P. aeruginosa*), 30 μg of chloramphenicol (an antibacterial agent selective for the Gram positive species *B. subtilis*), and 20 μg of nystatin (an antifungal agent).

No.	Bacteria			Fungi		
	E coli	*B sub*	*P aer*	*C alb*	*T ment*	*C res*
 **5.16**$^{+}$	4	3	1	10	22	10
 **5.21**$^{\#}$	-¶	-	-	-	-	-
 **5.28**$^{+}$	3	10	-	15	10	15
 **5.31**$^{+}$	-	-	-	-	1	-
 **5.37**$^{+}$	2	10	1	1	6	3
 **5.41**$^{+}$	-	-	-	-	3	-
Gentamycin	10		10			
Chloramphenicol		13				
Nystatin				10	7	12

$^{+}$ Activity tested in the solvent system and sample concentration specified in Table 5.1.
$^{\#}$ Activity tested in solvent system A at 10 mg mL^{-1}.
¶ No activity observed.

Table 5.3. Activity of **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41** against selected microbial species.

None of the 5-(hydroxymethyl)pyrrole-based compounds (**5.21**, **5.31** or **5.41**) displayed antibacterial activity, which suggests that compounds based on this structural motif are not effective bacterial growth inhibitors. The aromatic amines **5.16**, **5.28** and **5.37** displayed

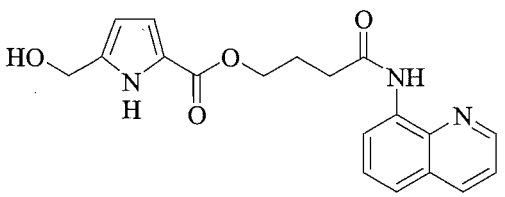
mild activity against *E. coli* and *P. aeruginosa*, but it was noticeably less than that elicited by gentamycin. Activity similar to that recorded for chloramphenicol against *B. subtilis* was displayed by the heterocyclic amines **5.28** and **5.37**, whereas the carbocyclic amine **5.16** was less active. The 5-(hydroxymethyl)pyrrole **5.21** was totally inactive against all fungal species. The other pyrrolic compounds, **5.31** and **5.41**, were less active against *T. mentagrophytes* than nystatin, and they did not inhibit *C. albicans* and *C. resinae* growth. Amines **5.16**, **5.28** and **5.37** were active against all fungal species, with **5.37** being noticeably weaker than **5.16** and **5.28**. Amine **5.28** displayed similar activity to nystatin against the three fungal species. Interestingly, the carbocycle **5.16** was comparable to nystatin for *C. albicans* and *C. resinae* growth inhibition, whereas its inhibition of *T. mentagrophytes* growth was three times higher than nystatin. Overall, compounds **5.21**, **5.31** and **5.41** were poor antimicrobial agents.

5.7.4 Determination of the binding constant of 5.31 to DNA

The relatively low activity displayed by **5.31** against the P388 cell line (see Table 5.1) could have resulted from a low affinity for DNA. Baguley *et al*[20] reported a method for determining the DNA binding activity of a compound, relative to ethidium bromide (**1.56**, Figure 5.9), by measurement of the competition for DNA binding sites. Compound **5.31** was submitted for analysis,[¶] and it had a 3,000-fold lower affinity for poly[dA-dT] (4.6 x 10^3 M^{-1}) and poly[dG-dC] (3.0 x 10^3 M^{-1}) than **1.56** (1.1 x 10^7 M^{-1}), *i.e.* **5.31** had a relatively low affinity for DNA. The equations in reference 20 were used by the experimentalist[¶] to calculate the binding constants for **5.31**, however the endpoint of the assay (20% displacement of **1.56** by **5.31**) was different to that typically used (50% displacement) due to the low solubility of **5.31** in the assay.[21] The binding mode of **5.31** to DNA is not determined in this assay, as both groove binding agents and intercalators compete with **1.56**.[22] A separate assay which determines whether intercalation is occurring by the unwinding of DNA[5] could not be performed due to the poor solubility of **5.31** in the

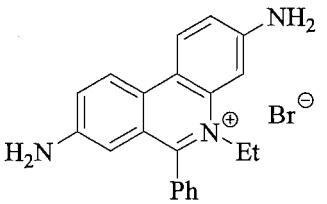
[¶] The author would like to thank Professor Bruce Baguley at the Auckland Cancer Society Research Centre for performing the assays, and calculating the binding constants.

assay.[21] However, **5.31** is unlikely to bind DNA as a result of intercalation, due to the lack of a tricyclic aromatic ring system.



5.31

K (poly[dA-dT]) = 4.6 x 10^3 M^{-1}
K (poly[dG-dC]) = 3.0 x 10^3 M^{-1}



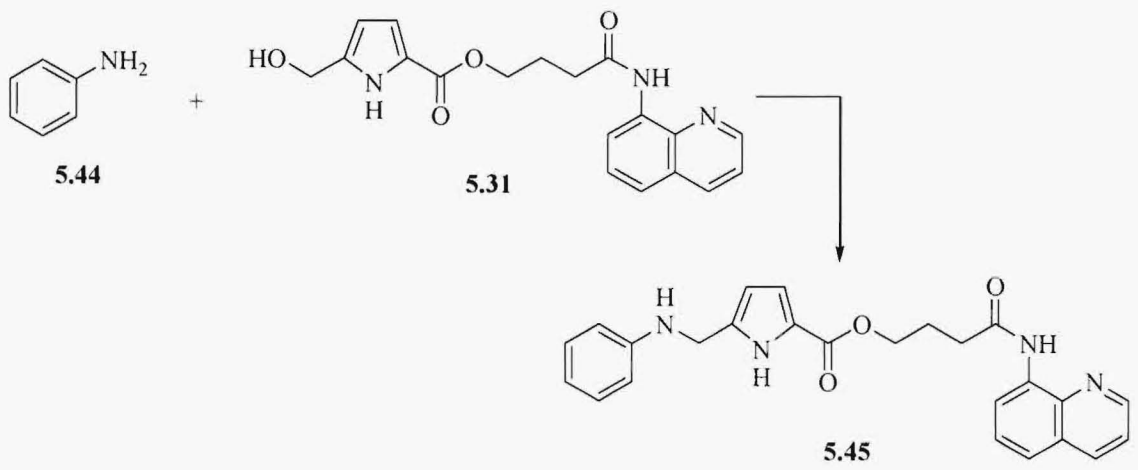
1.56

K (poly[dA-dT] or poly[dG-dC]) = 1.1 x 10^7 M^{-1}

Figure 5.9. DNA binding assay results for **5.31** and the classical intercalator **1.56**.

5.8 DETERMINATION OF THE ABILITY OF 5.31 TO ALKYLATE AN AROMATIC AMINE

The alkylating activity displayed by compounds of type **5.9** was reliant on the formation of a covalent bond to DNA. The mild activity of **5.21** and **5.31** against the P388 cell line could have resulted from the inability of the 5-(hydroxymethyl)pyrrole functionality to alkylate the genetic material. A model system was devised to elucidate whether **5.31** could alkylate aniline (**5.44**, Scheme 5.12) to give adduct **5.45**. This system was postulated to mimic the reaction between DNA nucleophiles and compounds of type **5.9** (see Scheme 5.2). Two equivalents of **5.44** were added to one equivalent of **5.31** in acetonitrile, and incubated at 37°C for 48 h. The first equivalent of **5.44** was added to induce azafulvene formation by deprotonation of the pyrrole ring nitrogen (see Scheme 5.2). Nucleophilic attack by the second equivalent of **5.44** upon the azafulvene would result in adduct **5.45**.



Scheme 5.12. Reaction of aniline (**5.44**) and **5.31** to afford **5.45**.

The HPLC traces for separate samples of **5.31** and **5.44** are displayed in Figure 5.10. The peaks below 5 min, and between 33-34 min, were artefacts of the solvent system used for HPLC analysis (see Experimental for details).

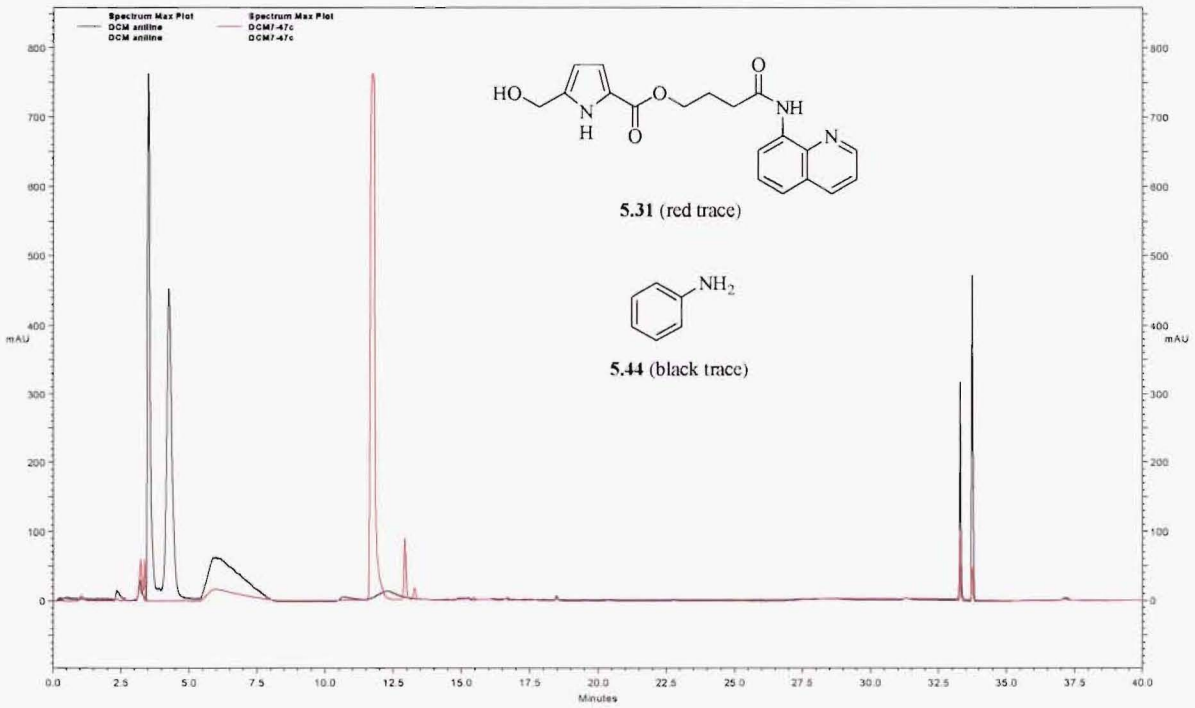


Figure 5.10. HPLC analysis of separate samples of **5.31** (red trace) and **5.44** (black trace).

Progress of the reaction between **5.31** and **5.44** was monitored by reverse-phase HPLC analysis after 0 h (blue trace), 24 h (green trace) and 48 h (pink trace) (Figure 5.11). A large peak eluted at approximately 12 min in all three HPLC analyses (*cf* trace for **5.31** in Figure 5.10, major peak elutes at approximately 12 min). After 24 h, this large peak was collected and analysed by mass spectrometry. The base peak in the spectrum had a mass of 354.1 g mol^{-1}, corresponding to protonated **5.31**. Analysis by mass spectrometry of the minor peaks at approximately 13 and 17.5 min revealed that they were unknown byproducts, *i.e.* adduct **5.45** was not being formed.

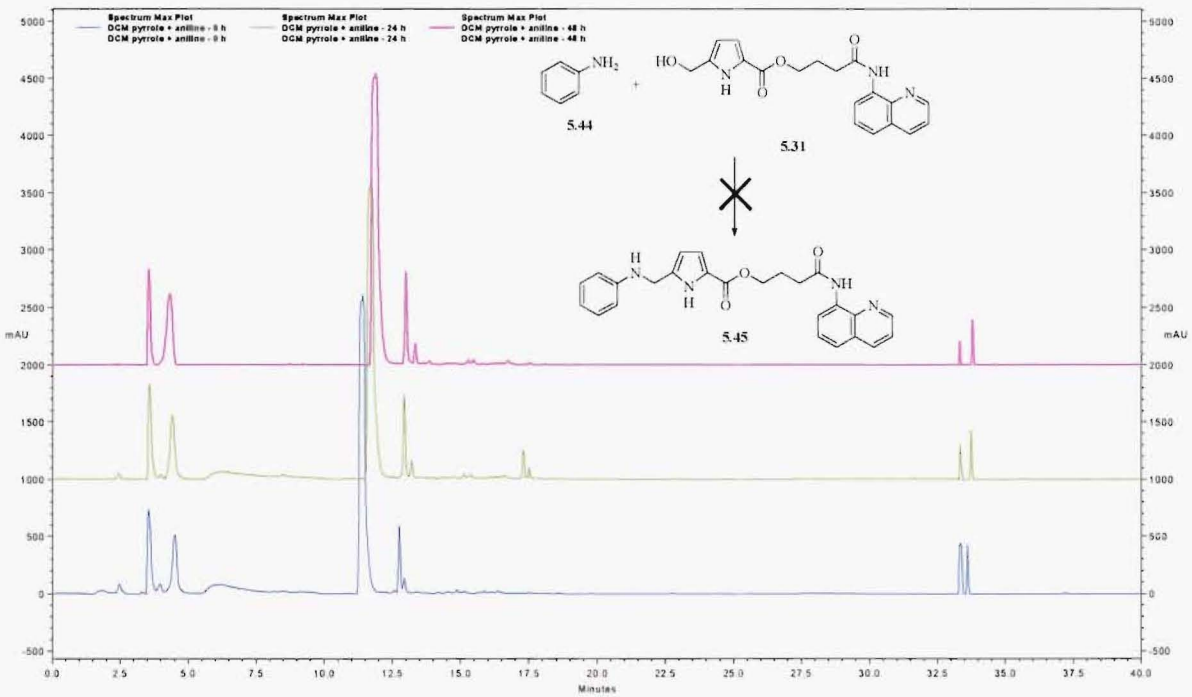


Figure 5.11. Traces from the HPLC analysis of the reaction between **5.44** and **5.31** after 0 h (blue), 24 h (green) and 48 h (pink).

These findings suggested that 5-(hydroxymethyl)pyrroles of type **5.9** are not reactive enough, under mild conditions, to undergo nucleophilic substitution of the hydroxyl group by an aromatic amine. As a result, this scaffold may not effect DNA alkylation.

5.9 CONCLUSION AND FUTURE WORK

The 5-(hydroxymethyl)pyrrole scaffold was used as the basis from which a new class of putative DNA alkylating agents, of type **5.9**, were developed. Model synthetic studies yielded **5.21** and **5.31**, which possess bicyclic naphthyl and quinolyl ring systems respectively as intercalator surrogates. However, the attempted preparation of **5.22** and **5.32**, which incorporate anthryl and acridinyl intercalating moieties respectively, was unsuccessful due to low yields and difficult purification. Preparation of **5.36**, a proposed DNA alkylator whose projected mode of binding to the genetic material was by threading intercalation, was also unsuccessful.

The biological activity of the pyrroles **5.21** and **5.31**, the aromatic amine starting materials **5.16** and **5.28** (used in the preparation of **5.21** and **5.31** respectively), a representative tricyclic structure **5.37**, and the pyrrole substructure analogue **5.41** was tested. Amine **5.16** was slightly less active against the P388 murine leukaemia cell line than **5.21**, whereas amine **5.28** was 3.5-fold more effective than **5.31**. Diamine **5.37** was noticeably more active than all the other compounds in the cancer cell line assay. Compound **5.41** was inactive against the cancer cell line. None of the compounds demonstrated antiviral activity. Compound **5.21** showed no antimicrobial activity, whereas **5.31** and **5.41** displayed only minimal antifungal activity. The aromatic amines **5.16**, **5.28** and **5.37** all showed antimicrobial activity to some degree. Pyrrole **5.31** displayed a low affinity for oligonucleotides, in comparison to the classical intercalator ethidium bromide, in a DNA binding assay. A model system, whereby **5.31** was incubated with aniline, was designed to mimic the reaction of a pyrrole-based alkylating agent with a DNA nucleophile. However, analysis by HPLC and mass spectrometry revealed that no reaction occurred between **5.31** and aniline.

Future work in this area would initially be centred on modifying the synthetic route, so that the tricyclic structure would be introduced at a later stage to overcome any problems with solubility and purification. Also, alterations to the 5-(hydroxymethyl)pyrrole functionality to introduce better leaving groups, and hence enhance azafulvene formation, could result in

more facile DNA alkylation. Once the optimal synthetic route is determined, a new series of structures would be prepared, and then assayed against the biological targets.

5.10 REFERENCES FOR CHAPTER FIVE

1. Altman, R.; Sarg, M. J. *The Cancer Dictionary*; Facts On File, Inc.: New York, 1992.

2. Niculescu-Duvaz, I.; Baracu, I.; Balaban, A. T. In *The Chemistry of Antitumour Agents*; Wilman, D. E. V., Ed.; Blackie & Son Ltd: Glasgow and London, 1990, pp. 63-130.

3. Anderson, W. K.; Corey, P. F. *J. Med. Chem.* **1977**, *20*, 812.

4. Sigurdsson, S. T.; Rink, S. M.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 12633.

5. Atwell, G. J.; Fan, J.-Y.; Tan, K.; Denny, W. A. *J. Med. Chem.* **1998**, *41*, 4744.

6. Hopkins, P. B. *Adv. DNA Sequence Specific Agents* **1996**, *2*, 217.

7. Woo, J.; Sigurdsson, S. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 3407.

8. Ortega, M. J.; Zubia, E.; Carballo, J. L.; Salva, J. *Tetrahedron* **1997**, *53*, 331.

9. Nabbs, B. K.; Abell, A. D. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 505.

10. Hubbard, J. L.; Carl III, J. M.; Anderson, G. D. *J. Heterocyclic Chem.* **1992**, *29*, 720.

11. Beckwith, A. L. J.; Boate, D. R. *J. Org. Chem.* **1988**, *53*, 4339.

12. Defacqz, N.; Touillaux, R.; Cordi, A.; Marchand-Brynaert, J. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2632.

13. Diago-Meseguer, J.; Palomo-Coll, A. L.; Fernandez-Lizarbe, J. R.; Zugaza-Bilbao, A. *Synthesis* **1980**, 547.

14. Atwell, G. J.; Bos, C. D.; Baguley, B. C.; Denny, W. A. *J. Med. Chem.* **1988**, *31*, 1048.

15. Von Hoff, D. D.; Myers, J. W.; Kuhn, J.; Sandbach, J. F.; Pocelinko, R.; Clark, G.; Coltman Jr., C. A. *Cancer Res.* **1981**, *41*, 3118.

16. Cain, B. F.; Atwell, G. J. *Eur. J. Cancer* **1974**, *10*, 539.

17. Carlson, C. B.; Beal, P. A. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1979.

18. Pitzer, N. W.; Ray, F. E. *J. Pharm. Sci.* **1968**, *57*, 348.

19. Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 589.

20. Baguley, B. C.; Denny, W. A.; Atwell, G. J.; Cain, B. F. *J. Med. Chem.* **1981**, *24*, 170.

21. Baguley, B. C., personal communication.

22. Baguley, B. C. *Cell. Mol. Biochem.* **1982**, *43*, 167.

CHAPTER SIX

EXPERIMENTAL

6.1 GENERAL METHODS AND EXPERIMENTAL PROCEDURES

Melting Points

All melting points were obtained on an Electrothermal apparatus and are uncorrected.

Nuclear Magnetic Resonance

Proton NMR spectra were obtained on a Varian Inova spectrometer, operating at 500 MHz. Carbon NMR spectra were obtained on a Varian Unity 300 spectrometer, operating at 75 MHz, with a delay (D_1) of 1 s. All spectra were obtained at 23°C. Chemical shifts are reported in parts per million (ppm) on the δ scale. Solvents used for NMR analysis (reference peaks listed) included: DMSO-d_6 ($C\underline{H}D_2SOCD_3$ at δ_H 2.60 ppm, $(\underline{C}D_3)_2SO$ at δ_C 39.6 ppm); $CDCl_3$ ($C\underline{H}Cl_3$ at δ_H 7.25 ppm, $\underline{C}DCl_3$ at δ_C 77.0 ppm); CD_3OD ($C\underline{H}D_2OD$ at δ_H 3.30 ppm, $\underline{C}D_3OD$ at δ_C 49.3 ppm); CD_3CN ($C\underline{H}D_2CN$ at δ_H 2.00 ppm, $\underline{C}D_3CN$ at δ_C 1.3 ppm); acetone-d_6 ($C\underline{H}D_2COCD_3$ at δ_H 2.17 ppm, $(\underline{C}D_3)_2CO$ at δ_C 29.2 ppm). Two-dimensional NMR experiments included COSY, HSQC, HMBC and CIGAR, and all were obtained on the Varian Inova spectrometer operating at 500 MHz. The HSQC, HMBC and CIGAR experiments were all obtained with a delay (D_1) of 1 s.

Infrared Spectroscopy

Infrared spectra were obtained using a Shimadzu 8201PC series FTIR interfaced with an Intel 486 PC operating Shimadzu's HyperIR software. Spectra were obtained in either $CHCl_3$ (solution phase) or solid KBr (diffuse reflectance).

Small Molecule Mass Spectrometry

Electron impact (EI) mass spectra were detected on a Kratos MS80 RFA mass spectrometer operating at 4000 V (accelerating potential) and 70 eV (ionisation energy). The source temperature was 200-250°C. Electrospray ionisation (ESI) mass spectra were detected on a micromass LCT TOF mass spectrometer, with a probe voltage of 3200 V, temperature of 150°C and a source temperature of 80°C. Direct ionisation used 10 μL of a

10 μg mL^{-1} solution, using a carrier solvent of 50% acetonitrile/H_2O at a flow rate of 20 μL min^{-1}. Ionisation was assisted by the addition of 0.5% formic acid.

Liquid Chromatography Mass Spectrometry

Inhibitor-enzyme samples were analysed initially by electrospray ionisation mass spectrometry (ESI) using a Micromass LCT mass spectrometer scanning at 100-2500 atomic mass units (scans every 0.9 s, 0.1 s delay). The source was at 80°C (ESI probe = 150°C, nebuliser = 190 L hr^{-1}, desolvation = 350 L hr^{-1}). Analysis by liquid chromatography mass spectrometry involved the use of a Waters 2790 LC, which was coupled to the Micromass LCT spectrometer mentioned above, with elution through a Waters 996 PDA Zorbax C3 column (150 mm x 2 mm x 5 μm, flow 0.2 mL min^{-1}). The mobile phase used a gradient of 100% [water/0.5% formic acid] to 40% [water/0.5% formic acid]/60% acetonitrile over 80 min, then to 100% acetonitrile over 5 min, held at 100% acetonitrile for 10 min, then back to 100% [water/0.5% formic acid] over 5 min. Data was processed with Maxent® software.

High Performance Liquid Chromatography

Analytical HPLC was performed on a Shimadzu LC-10AD VP liquid chromatograph coupled to a SIL-10A VP autoinjector, a CTO-10A VP column oven set to 40°C and a SPD-M10A VP photodiode array detector. The system was controlled by Shimadzu CLASS-VP (Version 5.02) software. Periodically, microfractions were collected for analysis by mass spectrometry. For reverse phase HPLC a Phenomenex Prodigy C18 5-ODS (3μ, 250 x 4.6 mm) column was used with a flow rate of 1 mL min^{-1} with a standard HPLC solvent gradient system that comprised of variable concentrations of H_2O (Milli-Q) containing 0.05% trifluoroacetic acid (TFA) and acetonitrile (HPLC grade). The gradient consisted of a 40 min run with the following steps – 2 min of 90% [water/0.05% TFA]/10% acetonitrile, followed by a linear gradient to 25% [water/0.05% TFA]/75% acetonitrile over 12 min, maintained at 25% [water/0.05% TFA]/75% acetonitrile for 10 min then a linear gradient to 100% acetonitrile over 2 min. This was held for 4 min then returned to 90% [water/0.05% TFA]/10% acetonitrile over 2 min. The 90% [water/0.05% TFA]/10% acetonitrile was maintained for 8 min to allow the column to re-equilibrate.

Microanalysis

Microanalysis was performed at the University of Otago Microanalytical Laboratory. All reported values are within ±0.4% of the calculated value.

Reagents, solvents and laboratory methodology

Oven-dried glassware was used in all reactions carried out under an inert atmosphere (either dry dinitrogen or argon). All starting materials and reagents were obtained commercially unless otherwise stated. "Removal of the solvent (or volatiles) by evaporation under reduced pressure" refers to the process of bulk solvent removal by rotary evaporation (low vacuum pump) followed by application of high vacuum (oil pump) for a minimum of 30 min. Analytical thin layer chromatography (TLC) was performed on plastic-backed Merck Keiselgel KG60F_{254} silica plates, and visualised using short wave ultraviolet light, ninhydrin or Mary's dip. Flash chromatography was performed using 230-400 mesh Merck Silica Gel 60 following established guidelines[1] under positive pressure. Kugelrohr distillations were performed under high vacuum (oil pump) with the slow application of heat to the specified temperature. THF and diethyl ether were distilled from sodium benzophenone ketyl under an inert atmosphere immediately prior to use. Dichloromethane, 1,2-dichloroethane, benzene and toluene were distilled from calcium hydride under an inert atmosphere. Petroleum ether refers to the fraction collected between 60-70°C. Ethyl acetate and petroleum ether were distilled from calcium hydride prior to their use in flash chromatography. DMF was dried by placing over 4 Å molecular sieves, applying a high vacuum for 15 min, then flushed briefly with an inert atmosphere. This process was repeated twice more over 24 h, after which the DMF was stored over 4 Å molecular sieves under an inert atmosphere. All other reagents and solvents were purified prior to use using literature procedures.[2]

General Procedure A: Acetylation of amines with acetyl chloride

A stirred suspension of the aniline in dry acetyl chloride (~0.35 M) under an inert atmosphere was refluxed for 15 min, after which it was cooled to r.t. and the volatiles were removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure B: Amide hydrolysis with 4:1 ethanol/5 M aqueous sodium hydroxide[3]

A stirred solution of the acetamide in 4:1 ethanol/5 M aqueous sodium hydroxide (~0.1 M) was refluxed for 1 h, after which it was cooled to r.t. and the solvent was removed by rotary evaporation. See individual experiments for details.

General Procedure C: Nitroaniline reductions with platinum oxide

A vigorously stirred suspension of the nitroaniline and platinum oxide (approximately 3:1 w/w ratio of starting material to catalyst), in dry ethanol (~0.025 M) at r.t., was evacuated under reduced pressure (low vacuum pump), then flushed with hydrogen gas. This process was repeated twice more, and the suspension was then left to stir under hydrogen for the specified time. The catalyst was removed by filtration of the reaction mixture through Celite and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure D: Benzimidazole preparation with polyphosphoric acid[4]

A stirred mixture of the diamine (1 equiv) and the pyridine derivative (1 equiv), in freshly prepared polyphosphoric acid[5] (~0.4 M) under an inert atmosphere, was heated to 170-180°C for 5 h. The reaction mixture was then poured onto ice (20 g), basified to pH ~8 with 28% aqueous ammonium hydroxide, and the resulting solid was collected by filtration. See individual experiments for details.

General Procedure E: Benzimidazole preparation with nitrobenzene[6]

To a stirred solution of the diamine (1 equiv) in dry nitrobenzene (~0.2 M) at r.t. under an inert atmosphere was added 2-pyridinecarboxaldehyde (1 equiv). The resulting solution

was warmed to 150°C for 18 h, after which it was cooled to r.t. and the solvent was removed by Kugelrohr distillation (up to 160°C). See individual experiments for details.

General Procedure F: Hydrolysis of formylpyrrole esters with potassium hydroxide

A stirring solution of the formylpyrrole (1 equiv) and potassium hydroxide (4 equiv) in water (~0.2 M) was heated to 40-50°C for 2 h. After cooling to r.t., the solution was acidified to pH ~1 with concentrated hydrochloric acid and extracted with ether (6 x 20 mL). The combined ethereal fractions were washed with water (2 x 20 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure G: Acylation of ethyl 1*H*-pyrrole-2-carboxylate (3.8) with an acid chloride[7]

A solution of ethyl 1*H*-pyrrole-2-carboxylate (**3.8**,[8] 1 equiv) in 1,2-dichloromethane (~0.6 M) was added dropwise over 5 min to a stirred suspension of the acid chloride (2 equiv) and zinc chloride (2 equiv) in 1,2-dichloromethane (~1.2 M), which had been cooled to 0°C under an inert atmosphere. The suspension was heated to 50°C for 1 h, after which the resulting solution was poured onto ice-water and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (40 mL), and the combined organic fractions were washed with aqueous saturated sodium hydrogen carbonate (30 mL), aqueous saturated brine (30 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure H: Hydrolysis of 5-acylpyrrole esters with potassium hydroxide

A stirred solution of the 5-acylpyrrole (1 equiv) and potassium hydroxide (4 equiv) in 2:1 water/THF (~0.15 M) was heated to 40-50°C for 2 h. After cooling to r.t., the solution was washed with ether (20 mL), acidified to pH ~1 with concentrated hydrochloric acid, and extracted with ether (5 x 20 mL). The combined ethereal fractions were dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure I: Couplings using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/1-hydroxybenzotriazole hydrate[9]

To a stirred solution of the pyrrole acid (1 equiv) and the amino acid ester hydrochloride (1.1 equiv) in dry dichloromethane (~0.1 M), at r.t. under an inert atmosphere, were added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.3 equiv), 1-hydroxybenzotriazole hydrate (1.5 equiv) and *N,N*-diisopropylethylamine (1.1 equiv). After 16 h, the solution was diluted with dichloromethane (10 mL), washed with 3 M aqueous hydrochloric acid (2 x 10 mL), water (2 x 10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

Modified General Procedure I

The reaction was performed in the manner described in General Procedure I, except that the combined aqueous washings were back-extracted with dichloromethane (2 x 10 mL), and the combined organic fractions were then dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure J: Zinc borohydride reductions[10]

A stirred solution of the pyrrole (1 equiv), dissolved in dry ether (~0.1 M) under an inert atmosphere, was cooled to 0°C. Zinc borohydride (2.2 equiv of a 0.14 M solution in ether) was added, and the resultant solution was stirred at 0°C for 1 h. Water (1 mL), and then 10% aqueous acetic acid (1 mL), were carefully added to quench the reaction. The separated aqueous phase was extracted with dichloromethane (2 x 10 mL), and the combined organic phases were washed with water (2 x 10 mL), saturated aqueous brine (10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure K: Lithium borohydride reductions

A stirred solution of the pyrrole (1 equiv), dissolved in dry THF (~0.03 M) under an inert atmosphere, was cooled to -78°C (dry ice/acetone). Lithium borohydride (2 equiv) was added, and the resultant solution was stirred at -78°C for 1 h, then warmed to r.t. and

stirred for an additional 1 h. Water (10 mL) was carefully added to quench the reaction. The solution was extracted with ethyl acetate (3 x 10 mL), the combined organic phases were washed with saturated aqueous brine (10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

Modification to General Procedure K

The reaction was performed in the manner described in General Procedure I, except that dry ether was used in place in dry THF, and the reaction was stirred at -78°C for 1 h before workup. See individual experiments for details.

General Procedure L: Esterification of carboxylic acids with diazomethane

To a stirred solution of the acid (1 equiv) in dry THF (~0.075 M) was added a 12-fold excess of diazomethane in ether dropwise. After stirring at r.t. for 15 h, the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure M: Couplings using benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate[11]

To a stirred solution of the pyrrole acid (1 equiv) and the amino acid ester hydrochloride (1.1 equiv), or the free amine of the amino acid ester (1.1 equiv), in dry dichloromethane (~0.1 M) at r.t. under an inert atmosphere (shielded from light) were added benzotriazol-1-yloxytris(dimethyl-amino)phosphonium hexafluorophosphate (1.1 equiv) and *N,N*-diisopropylethylamine (Hünig's base, 1.1 equiv). After 16 h, the solution was diluted with ethyl acetate (15 mL), washed with 1 M aqueous hydrochloric acid (10 mL), aqueous saturated sodium hydrogen carbonate (10 mL), aqueous saturated brine (10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure N: Pyrrolizinone formation from 5-formylpyrroles with hydrocinnamoyl chloride

To a stirred solution of the 5-formylpyrrole (1 equiv) in dry dichloromethane (~0.025 M), at r.t. under an inert atmosphere, was added 4-(dimethylamino)pyridine (0.1 equiv),

followed by the dropwise addition of *N,N*-diisopropylethylamine (2 equiv). After 5 min, hydrocinnamoyl chloride (2 equiv) dissolved in dry dichloromethane (2 mL) was added dropwise over 5 min, and the resultant solution was stirred for 24 h. Ethyl acetate (10 mL) was added and the organic solvents were washed with 10% aqueous citric acid (10 mL), water (10 mL), saturated aqueous brine (10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure O: Esterification of dipeptide acids with methanol

A stirred solution of the dipeptide carboxylic acid (1 equiv) was suspended in dry methanol (~0.2 M) at r.t. under an inert atmosphere. Oxalyl chloride (1.1 equiv) was added dropwise, followed by DMF (2 drops). The resultant solution was refluxed for 3 h, after which the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

General Procedure P: Reaction of aromatic amines with succinic anhydride in refluxing toluene[12]

A stirred solution of the aromatic amine (1 equiv) and succinic anhydride (1 equiv) in dry toluene (~0.25 M) under an inert atmosphere was refluxed for 15 min. After cooling to r.t., the solid was collected by filtration and washed with cold petroleum ether. The material was dissolved in ethyl acetate (60 mL) and 5% aqueous sodium hydroxide (60 mL), the layers were separated, and the organic phase was extracted with 5% aqueous sodium hydroxide (20 mL). The combined aqueous fractions were acidified to pH ~1 with concentrated hydrochloric acid, and the resultant solid was allowed to stand for 3 h, after which it was collected by filtration, washed with water (10 mL) and dried under reduced pressure. See individual experiments for details.

Modification to General Procedure P

The reaction was performed in the manner described in General Procedure P, except that the reaction mixture was refluxed for 1 h.

General Procedure Q: Succinimide formation from *N*-arylsuccinamic acids[13]

A mixture of the *N*-arylsuccinamic acid (1 equiv) and anhydrous sodium acetate (0.33 equiv) in dry acetic anhydride (~ 0.25 M), under an inert atmosphere, was heated to 70-80°C for 4.5 h, after which it was cooled to r.t., poured onto water (60 mL), and extracted with dichloromethane (3 x 20 mL). The combined organic fractions were washed with aqueous saturated ammonium chloride (20 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. Residual solvent was removed by Kugelrohr distillation (up to 140°C) from the desired product. See individual experiments for details.

General Procedure R: Succinimide reduction/ring opening with sodium borohydride[14]

To a stirred suspension of the succinimide (1 equiv) in 6:1 *iso*-propanol/water (~0.1 M) was added sodium borohydride (2.5 equiv), and the resulting suspension was stirred at r.t. for 17 h. See individual experiments for details.

Modification to General Procedure R

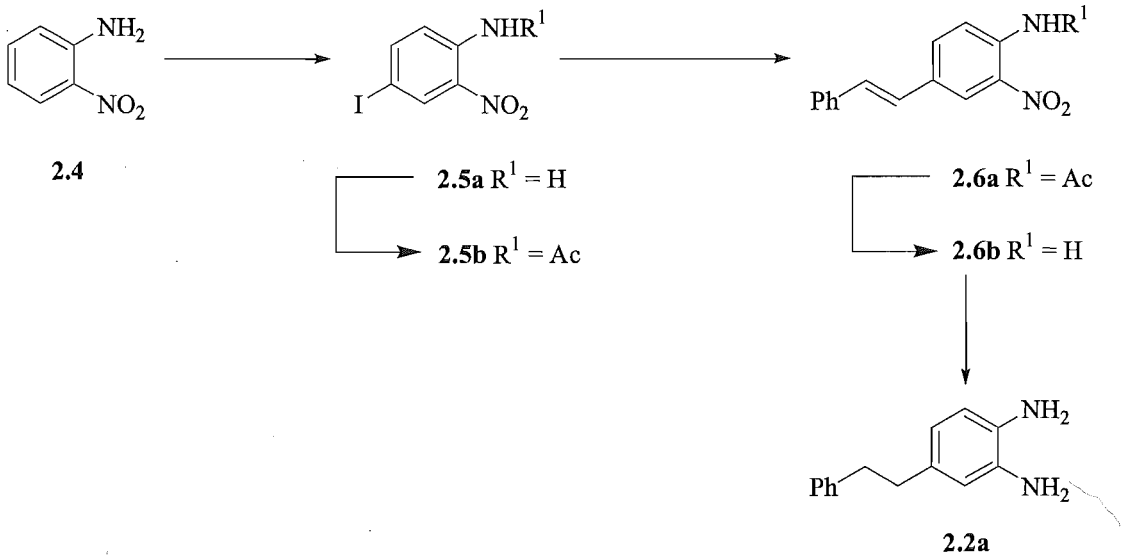
The reaction was performed in the manner described in General Procedure P, except that the disuccinimide was treated with 5 equiv of sodium borohydride.

General Procedure S: Couplings using bis(2-oxo-3-oxazolidinyl)phosphinic chloride[15]

To a stirred suspension of the pyrrole acid **3.9a** (1 equiv), alcohol (1.5 equiv) and bis(2-oxo-3-oxazolidinyl)phosphinic chloride (2 equiv) in dry dichloromethane (~0.07 M), at r.t. under an inert atmosphere, was added dry triethylamine (2 equiv). The resulting suspension was stirred for 64 h, after which it was diluted with ethyl acetate (20 mL), washed with 10% aqueous sodium bicarbonate (10 mL), saturated aqueous brine (10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. See individual experiments for details.

6.2 EXPERIMENTAL WORK DESCRIBED IN CHAPTER TWO

6.2.1 Preparation of the diamine intermediate 2.2a



4-Iodo-2-nitroaniline (2.5a)[16]

To a stirred suspension of 2-nitroaniline **2.4** (1.50 g, 11 mmol, 1 equiv) in 30% aqueous acetic acid (50 mL) at r.t. was added pyridine iodine monochloride (2.62 g, 11 mmol, 1 equiv) in a portionwise fashion over 5 min. Stirring was continued for 30 min, after which the solid was collected by filtration and washed with water. The product was dried under reduced pressure (high vacuum) to give **2.5a** (2.78 g, 97%) as an orange solid.

mp 120-122°C (lit.[16] 119-121°C).

^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.95 (d, 1H, J = 8.8 Hz, H6), 7.63 (s(br), 2H, NH$_2$), 7.70 (dd, 1H, J = 2.0, 8.8 Hz, H5), 8.29 (d, 1H, J = 2.0 Hz, H3).

4-Iodo-2-nitroacetanilide (2.5b)

The aniline **2.5a** (2.0 g, 7.58 mmol) was treated with acetyl chloride by General Procedure A to give **2.5b** (2.12 mg, 91%) as a yellow solid.

mp 109-110°C (lit.[17] 112°C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.28 (s, 3H, COCH$_3$), 7.89 (dd, 1H, J = 2.0, 8.8 Hz, H5), 8.51 (d, 1H, J = 2.0 Hz, H3), 8.56 (d, 1H, J = 9.3 Hz, H6), 10.25 (s, 1H, NHCO).

(*E*)-2-Nitro-4-(2-phenylvinyl)acetanilide (2.6a)

To a stirred solution of the acetanilide **2.5b** (1.50 g, 4.90 mmol, 1 equiv), palladium acetate (110 mg, 0.49 mmol, 0.1 equiv) and tri-*o*-tolylphosphine (298 mg, 0.98 mmol, 0.2 equiv) in dry acetonitrile (110 mL), under an inert atmosphere at r.t., was added dry styrene (2.55 g, 24.5 mmol, 5 equiv) and dry triethylamine (5 mL). The resulting solution was refluxed for 3 h, after which the solvent was removed by evaporation under reduced pressure. The crude product was purified by flash chromatography (ethyl acetate/petroleum ether 2:3, then ethyl acetate, then ethyl acetate/acetone 1:1), followed by recrystallisation from ethyl acetate, to give **2.6a** (704 mg, 51%) as an orange solid.

mp 181-183°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.30 (s, 3H, $COCH_3$), 7.09 (AB_q, 2H, J = 16.6 Hz, CH=CH), 7.29 (t, 1H, J = 7.3 Hz, ArH), 7.37 (t, 2H, J = 7.6 Hz, ArH), 7.51 (d, 2H, J = 7.3 Hz, ArH), 7.79 (dd, 1H, J = 2.0, 8.8 Hz, H5), 8.31 (d, 1H, J = 2.0 Hz, H3), 8.77 (d, 1H, J = 8.8 Hz, H6), 10.32 (s(br), 1H, NHCO).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 24.9 ($COCH_3$), 122.3 (C6), 122.5 (C3), 125.0, 130.0 (CH=CH), 126.2, 127.8, 128.3, 135.8 (ArC), 132.4 (C5), 132.6 (C4), 132.7 (C1), 135.8 (C2), 168.5 ($COCH_3$).

FTIR (KBr, diffuse refraction method) cm^{-1} 1515.9, 1575.7, 1701.1, 3062.7, 3348.2.

HRMS (EI) 282.0996 (M^+). $C_{16}H_{14}N_2O_3$ requires 282.1004.

(*E*)-2-Nitro-4-(2-phenylvinyl)aniline (2.6b)

The acetanilide **2.6a** (600 mg, 2.13 mmol) was treated with 4:1 ethanol/5 M aqueous sodium hydroxide by General Procedure B. The resulting solid was washed with cold water (5 x 10 mL) and dried under reduced pressure (high vacuum) to give **2.6b** (510 mg, quantitative yield) as a dark red solid.

mp 203-205°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.14 (s(br), 2H, NH_2), 6.82 (d, 1H, J = 8.3 Hz, H6), 6.99 (s, 2H, CH=CH), 7.25 (m, 1H, ArH), 7.35 (t, 2H, J = 7.6 Hz, ArH), 7.48 (d, 2H, J = 7.3 Hz, ArH), 7.60 (dd, 1H, J = 2.2, 8.5 Hz, H5), 8.21 (d, 1H, J = 2.0 Hz, H3).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 119.2 (C6), 124.0 (C3), 126.3, 128.7 (CH=CH), 126.4, 127.7, 127.7, 137.0 (ArC), 127.1 (C1), 127.2 (C4), 133.2 (C5), 143.9 (C2).

FTIR (KBr, diffuse refraction method) cm^{-1} 1523.7, 1556.4, 1581.5, 1645.2, 3062.1, 3184.3, 3338.6, 3467.8.

HRMS (EI) 240.0901 (M^+). $C_{14}H_{12}N_2O_2$ requires 240.0899.

4-(2-Phenylethyl)benzene-1,2-diamine (2.2a)

The nitroaniline **2.6b** (110 mg, 0.46 mmol) was reduced with platinum oxide under hydrogen for 17 h by General Procedure C. Recrystallisation of the crude product from ethanol/water gave **2.2a** (93 mg, 96%) as a light purple solid.

mp 76-79°C.

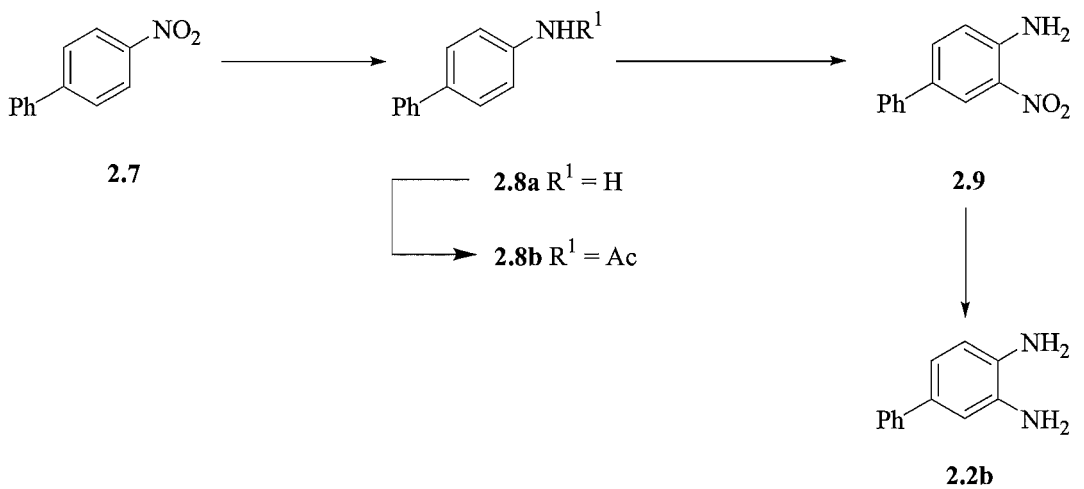
^1H NMR ($CDCl_3$, 500 MHz) δ 2.77, 2.85 (2 x m, 4H, $C\underline{H}_2C\underline{H}_2$), 6.54 (m, 2H, H3 plus H5 or H6), 6.63 (d, 1H, J = 7.8 Hz, H5 or H6), 7.18 (m, 3H, Ar$\underline{H}$), 7.27 (m, 2H, Ar$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 37.3, 38.2 ($\underline{C}H_2\underline{C}H_2$), 116.8, 117.0, 119.9 (C3, C5, C6), 125.7, 128.2, 128.4, 142.1 (Ar$\underline{C}$), 132.3, 134.1, 134.9 (C1, C2, C4).

FTIR (KBr, diffuse refraction method) cm^{-1} 1569.9, 2850.6, 2931.6, 3367.5.

HRMS (EI) 212.1318 (M^+). $C_{14}H_{16}N_2$ requires 212.1314.

6.2.2 Preparation of the diamine intermediate 2.2b



4-Aminobiphenyl (2.8a)

A vigorously stirred suspension of 4-nitrobiphenyl **2.7** (4.37 g, 21.9 mmol) and 10% palladium on carbon (approximately 1.50 g) in dry ethanol (45 mL) was evacuated under vacuum (low vacuum pump), then flushed with hydrogen gas. This process was repeated twice more, and the suspension was then left to stir at r.t. for 17 h. The catalyst was removed by filtration, and the solvent was removed by evaporation under reduced pressure to give **2.8a** (3.55 g, 96%) as an off-white solid that was not subjected to further purification.

mp 48-50°C (lit.[18] 53-54°C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.71 (s(br), 2H, N$\underline{H}_2$), 6.76 (d, 2H, J = 8.3 Hz, Ar$\underline{H}$), 7.26 (t, 1H, J = 7.3 Hz, Ar$\underline{H}$), 7.40 (m, 4H, Ar$\underline{H}$), 7.53 (d, 2H, J = 7.3 Hz, Ar$\underline{H}$).

4-Acetamidobiphenyl (2.8b)

The aminobiphenyl **2.8a** (3.53 g, 20.9 mmol) was treated with acetyl chloride by General Procedure A to give **2.8b** (4.41 g, quantitative yield) as a white solid.

mp 172-173°C (lit.[19] 171°C).

^{1}H NMR ($CDCl_3$ + 2 drops DMSO-d_6, 500 MHz) δ 1.90 (s, 3H, COC$\underline{H}_3$), 7.05 (t, 1H, J = 7.3 Hz, Ar$\underline{H}$), 7.16 (t, 2H, J = 7.6 Hz, Ar$\underline{H}$), 7.28 (m, 4H, Ar$\underline{H}$), 7.42 (d, 2H, J = 8.8 Hz, Ar$\underline{H}$), 9.21 (s(br), 1H, N$\underline{H}$CO).

4-Amino-3-nitrobiphenyl (2.9)[20]

To a stirred solution of the acetamidobiphenyl **2.8b** (500 mg, 1.95 mmol), in dry glacial acetic acid (4.8 mL) under an inert atmosphere at 70°C, was added dropwise over 5 min a solution of fuming nitric acid (0.32 mL) in dry glacial acetic acid (4.8 mL). After stirring at 70°C for 1 h, the solution was poured onto cold water (30 mL), and the resulting solid was collected by filtration, washed with cold water and dried under reduced pressure (high vacuum) to give 4-acetamido-3-nitrobiphenyl as a yellow solid. This material was treated with 4:1 ethanol/5 M aqueous sodium hydroxide by General Procedure B. The solid was collected by filtration and washed thoroughly with 10% aqueous sodium hydroxide. The product was dried under reduced pressure (high vacuum) to give **2.9** (384 mg, 76% yield over two steps) as a dark red solid.

mp 163-166°C (lit.[20] mp 167-169°C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.10 (s(br), 2H, NH_2), 6.89 (d, 1H, J = 8.8 Hz, H5), 7.33 (t, 1H, J = 7.3 Hz, ArH), 7.43 (t, 2H, J = 7.8 Hz, ArH), 7.55 (m, 2H, ArH), 7.64 (dd, 1H, J = 2.2, 8.5 Hz, H6), 8.37 (d, 1H, J = 2.0 Hz, H2).

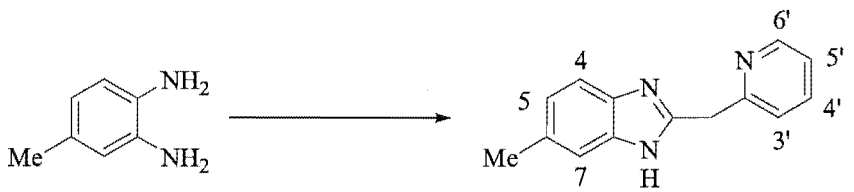
3,4-Diaminobiphenyl (2.2b)

The aminobiphenyl **2.9** (200 mg, 0.93 mmol) was reduced with platinum oxide and hydrogen for 6 h by General Procedure C. Recrystallisation of the crude product from benzene/petroleum ether gave **2.2b** (141 mg, 82%) as a brown solid.

mp 102-104°C (lit.[21] 103°C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.76 (d, 1H, J = 7.8 Hz, H5), 6.97 (m, 2H, H2 and H6), 7.26 (t, 1H, J = 7.3 Hz, ArH), 7.37 (t, 2H, J = 7.6 Hz, ArH), 7.51 (d, 2H, J = 7.3 Hz, ArH).

6.2.3 Preparation of the benzimidazole 2.1c



2.2c **2.1c**

5(6)-Methyl-2-(pyridin-2-ylmethyl)-1*H*-benzimidazole (2.1c)

Method A

3,4-Diaminotoluene **2.2c** (300 mg, 2.46 mmol, 1 equiv) was reacted with 2-pyridylacetic acid hydrochloride (426 mg, 2.46 mmol, 1 equiv) by General Procedure D. The crude product was purified by flash chromatography on silica (ethyl acetate, then methanol), followed by recrystallisation from ethyl acetate/petroleum ether, to give **2.1c** (274 mg, 50%) as a light brown solid.

mp 134-136°C

^{1}H NMR (CD_3OD, 500 MHz) δ 2.42 (s, 3H, C<u>H</u>$_3$), 4.38 (s, 2H, C<u>H</u>$_2$), 7.03 (dd, 1H, J = 1.5, 8.3 Hz, <u>H5(6)</u>), 7.30 (m, 2H, <u>H7</u> and <u>H5'</u>), 7.36 (m, 2H, <u>H4</u> and <u>H4'</u>), 7.78 (dt, 1H, J = 2.0, 7.8 Hz, <u>H3'</u>), 8.49 (d, 1H, J = 4.9 Hz, <u>H6'</u>).

FTIR (KBr, diffuse refraction method) cm^{-1} 1569.9, 1593.1, 2684.7, 2833.2, 2920.0, 3014.5.

HRMS (ES) 224.1183 (M^++H). $C_{14}H_{14}N_3$ requires 224.1188.

Method B

3,4-Diaminotoluene **2.2c** (300 mg, 2.46 mmol, 1 equiv) was reacted with 2-pyridylacetonitrile (290 mg, 2.46 mmol, 1 equiv) by General Procedure D to give **2.1c** (425 mg, 78% crude mass return) as a dark purple solid. No purification was attempted due to the adequate amount of pure **2.1c** obtained from Method A.

mp 127-132°C.

^{1}H NMR data was identical to that above.

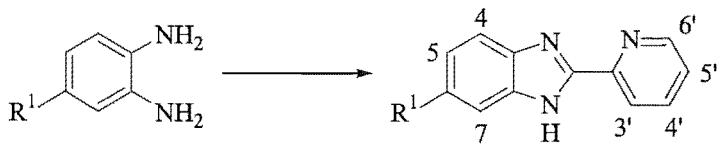
Method C

3,4-Diaminotoluene **2.2c** (100 mg, 0.82 mmol, 1 equiv) and 2-pyridylacetic acid hydrochloride (142 mg, 0.82 mmol, 1 equiv) in 4 M hydrochloric acid (5 mL), under an inert atmosphere, were heated to 120-130°C for 6 h. The reaction mixture was cooled to 0°C, and the solid was collected by filtration and dried under reduced pressure (high pressure) to give **2.1c** (dihydrochloride salt, 242 mg, quantitative mass return) as a grey solid.

^{1}H NMR (CD_3OD, 500 MHz) δ 2.33 (s, 3H, C<u>H</u>$_3$), 4.22 (s, 2H, C<u>H</u>$_2$), 7.05 (m, 2H), 7.21 (d, 1H, J = 7.8 Hz), 8.04 (m, 2H), 8.61 (m, 1H), 8.84 (m, 1H).

No further analytical data was obtained due to product degradation.

6.2.4 Preparation of the benzimidazoles 2.14a-c



2.2a $R^1 = Ph(CH_2)_2$
2.2b $R^1 = Ph$
2.2c $R^1 = Me$

2.14a $R^1 = Ph(CH_2)_2$
2.14b $R^1 = Ph$
2.14c $R^1 = Me$

5(6)-(2-Phenylethyl)-2-(pyridin-2-yl)-1*H*-benzimidazole (2.14a)

Diamine **2.2a** (80 mg, 0.38 mmol, 1 equiv) was treated with 2-pyridinecarboxaldehyde (40 mg, 0.38 mmol, 1 equiv) by General Procedure E. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 3:1) to give **2.14a** (48 mg, 43%) as a tan solid.

mp decomp. >35°C.

^{1}H NMR (CD_3CN, 500 MHz) δ 3.03, 3.10 (2 x m, 4H, CH_2CH_2), 7.23 (m, 2H, ArH and H5(6)), 7.32 (m, 4H, ArH), 7.49 (m, 2H, H7 and H5'), 7.61 (d, 1H, J = 8.3 Hz, H4), 7.96 (dt, 1H, J = 1.5, 7.8 Hz, H4'), 8.38 (d, 1H, J = 8.3 Hz, H3'), 8.71 (d, 1H, J = 4.9 Hz, H6').

^{13}C NMR (CD_3CN + 10 μL CF_3CO_2H, 75 MHz) δ 38.4 (2C, CH_2CH_2), 114.6 (C7), 115.0 (C4), 124.3 (C3'), 127.1, 129.4, 129.6, 142.3 (ArC), 129.1 (C5'), 129.6 (C5(6)), 130.6, 132.5 (C4a, C7a), 139.7 (C4'), 141.9 (C2'), 143.3 (C6(5)), 148.3 (C2), 152.0 (C6').

FTIR (KBr, diffuse refraction method) cm^{-1} 1569.9, 1596.9, 2858.3, 2929.7.

HRMS (EI) 299.1425 (M^+). $C_{20}H_{17}N_3$ requires 299.1423.

This product (20 mg, 0.07 mmol) was dissolved in ethyl acetate (5 mL), and dry hydrogen chloride gas was bubbled through the solution until a solid formed. The solvent was removed by evaporation under reduced pressure to give **2.14a** (dihydrochloride salt, 21 mg, 84%) as a tan solid.

mp 225-228°C.

^{1}H NMR (CD_3CN plus 2 drops DMSO-d_6, 500 MHz) δ 3.04, 3.16 (2 x m, 4H, CH_2CH_2), 7.24 (m, 1H, ArH), 7.31 (m, 4H, ArH), 7.49 (m, 1H, H5(6)), 7.70 (m, 2H, H7 and H5'), 7.81 (d, 1H, J = 8.3 Hz, H4), 8.16 (dt, 1H, J = 2.0, 7.8 Hz, H4'), 8.89 (d, 1H, J = 4.4 Hz, H6'), 9.03 (d, 1H, J = 8.3 Hz, H3').

5(6)-Phenyl-2-(pyridin-2-yl)-1*H*-benzimidazole (2.14b)

Diamine **2.2b** (100 mg, 0.54 mmol, 1 equiv) was treated with 2-pyridinecarboxaldehyde (58 mg, 0.54 mmol, 1 equiv) by General Procedure E. The crude material was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 3:1) to give **2.14b** (84 mg, 57%) as a tan solid. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **2.14b** dissolved in ethyl acetate.

mp 161-163°C (lit.[22] 164-165°C).

^{1}H NMR (CD_3CN, 500 MHz) δ 7.43 (m, 1H, ArH), 7.53 (m, 3H, ArH and H5'), 7.64 (m, 1H, H5(6)), 7.79 (m, 3H, ArH and H4), 7.99 (m, 2H, H7 and H4'), 8.43 (d, 1H, J = 7.8 Hz, H3'), 8.75 (d, 1H, J = 3.4 Hz, H6').

^{13}C NMR (CD_3CN + 10 μL CF_3CO_2H, 75 MHz) δ 113.1 (C7), 115.6 (C4), 124.4 (C3'), 128.0 (C5(6)), 128.5, 129.2, 130.2, 140.4 (ArC), 129.3 (C5'), 131.6 (C7a), 133.0 (C4a), 139.6 (C4'), 141.7 (C6(5)), 141.8 (C2'), 149.1 (C2), 152.0 (C6').

FTIR (KBr, diffuse refraction method) cm^{-1} 1560.3, 1595.0, 2360.7, 3058.9.

HRMS (EI) 271.1111 (M^+). $C_{18}H_{13}N_3$ requires 271.1110.

5(6)-Methyl-2-(pyridin-2-yl)-1*H*-benzimidazole (2.14c)

3,4-Diaminotoluene **2.2c** (125 mg, 1.02 mmol, 1 equiv) was treated with 2-pyridinecarboxaldehyde (110 mg, 1.02 mmol, 1 equiv) by General Procedure E. The crude material was purified by flash chromatography on silica (ethyl acetate/acetone, 5:1), followed by Kugelrohr distillation (up to 230°C, product collected in second bulb), which afforded a residue that was triturated with ether/petroleum ether to give **2.14c** (98 mg, 46%) as a tan solid.

mp 156-158°C (lit.[22] 159-160°C).

^{1}H NMR (CD_3CN, 500 MHz) δ 2.53 (CH_3), 7.17 (m, 1H, H5(6)), 7.49 (m, 2H, H7 and H5'), 7.60 (d, 1H, J = 8.3 Hz, H4), 7.96 (dt, 1H, J = 1.5, 7.8 Hz, H4'), 8.38 (dd, 1H, J = 1.0, 9.3 Hz, H3'), 8.71 (dd, 1H, J = 1.0, 4.9 Hz, H6').

^{13}C NMR (CD_3CN + 10 μL CF_3CO_2H, 75 MHz) δ 21.7 (CH_3), 114.6 (C7), 114.8 (C4), 124.2 (C3'), 129.0 (C5'), 129.9 (C5), 130.3, 132.5 (C4a, C7a), 139.3 (C6), 139.5 (C4'), 141.9 (C2'), 148.1 (C2), 152.0 (C6').

FTIR (KBr, diffuse refraction method) cm^{-1} 1569.9, 1593.1, 3051.2.

HRMS (EI) 209.0957 (M^+). $C_{13}H_{11}N_3$ requires 209.0953.

Micro. Calcd for $C_{13}H_{11}N_3$: C, 74.62; H, 5.30; N, 20.08. Found: C, 74.46; H, 5.22; N, 19.96.

6.2.5 α-Chymotrypsin assay and results

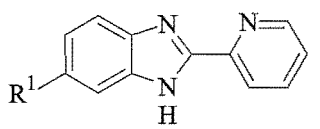
General Assay Conditions

The colorimetric-based method[23] was carried out in microtitre plates (twelve 1 x 8 well Titertek® microtitre strips), with absorbance readings taken in a BMG Labtechnologies Fluostar Galaxy 96-well plate reader. The enzyme and test solution were pre-incubated to ensure maximum inhibition before addition of the substrate, after which the optical density was measured at hourly intervals until the maximum measured absorbance exceeded 1.00 absorbance units (typically 2 or 3 h). A lack of colour development indicated inhibition of enzyme-catalysed 4-nitroaniline release. Control samples were included in all cases. Each sample was assayed in triplicate. Methanol was used to dissolve the proposed inhibitor and for sample dilution, as it is water soluble and directly compatible with the assay system.

α-Chymotrypsin Assay

Solutions of the benzimidazoles **2.14a-c** and **2.14a.2HCl** were made to 10 mM, 1.0 mM, 0.1 mM, 10 μM, 1.0 μM, 0.1 μM and 10 nM in methanol. Tris-HCl (50 μL of a 0.4 M solution in water, pH 7.6), inhibitor solution (50 μL), α-chymotrypsin (50 μL, Sigma ex-Bovine pancreas, 9 units mL^{-1} in 50 mM Tris-HCl, pH 7.6) and zinc chloride (50 μL of a 1.0 mM solution in distilled water) or EDTA disodium dihydrate (50 μL of a 5.0 mM solution in distilled water) were added to each well of the microtitre plate. Incubation at 37°C for 30 min was followed by the addition of *N*-succinyl-L-phenylalanine-4-nitroanilide (100 μL, 1 mg mL^{-1} solution in 50 mM Tris-HCl buffer, pH 7.6). The absorbance was read at 405 nm at hourly intervals until the maximum measured absorbance exceeded 1.00 absorbance units. Each inhibitor concentration was assayed in

triplicate and average absorbances were used to calculate the percentage (%) inhibition. The calculated percent inhibition values are a measure of the enzyme activity relative to that when no enzyme was present (no substrate turnover, 100% "inhibition") and when no inhibitor was present (full substrate turnover, 0% inhibition). To this end, sample blanks to determine the absorbance for each inhibitor concentration in the absence of enzyme were run concurrently, in which 50 mM Tris-HCl buffer (pH 7.6) replaced α-chymotrypsin. Maximum substrate turnover was determined by averaging the absorbance readings from six wells in which methanol replaced the inhibitor solution. All percentage (%) inhibition calculated values were rounded to the nearest 1%. The IC_{50} values were calculated by fitting the inhibitor concentration-percent inhibition data into Microsoft Excel®, with the inhibitor concentrations converted to their $\log_{10}$ values. Activity of the test compounds in the presence of EDTA was performed in an analogous manner, except that the zinc chloride solution was replaced by EDTA disodium dihydrate in distilled water (50 μL of a 5.0 mM solution in distilled water)

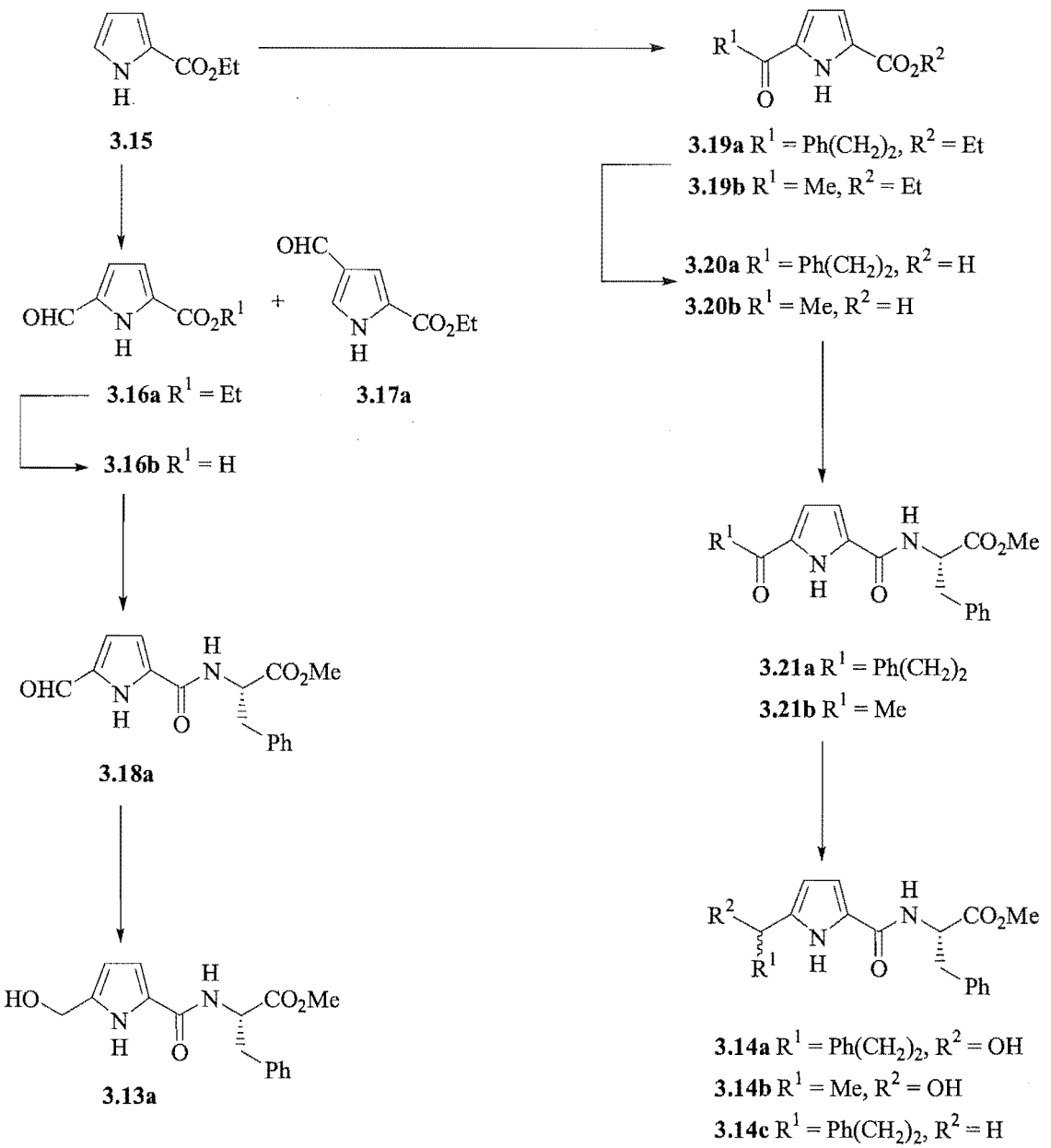


No.	R^1	IC_{50} (μM)	
		Zn^{2+}	EDTA
2.14a	$Ph(CH_2)_2$	81	>17
2.14b	Ph	>17	>17
2.14c	Me	>167	681
2.14a.2HCl	$Ph(CH_2)_2$	82	>17

Table 6.1. IC_{50} values for **2.14a-c** and **2.14a.2HCl** in the presence of Zn^{2+} or EDTA.

6.3 EXPERIMENTAL WORK DESCRIBED IN CHAPTER THREE

6.3.1 Preparation of the 5-(hydroxymethyl)pyrrole 3.13a and the C5-(hydroxyalkyl)pyrrole 3.14a-b



Ethyl 5-formyl-1*H*-pyrrole-2-carboxylate (3.16a)[24]

Phosphorous oxychloride (6.89 g, 45 mmol, 1.1 equiv) was added dropwise over 15 min to stirred DMF (3.29 g, 45 mmol, 1.1 equiv) cooled to 10-20°C under a nitrogen atmosphere. 1,2-Dichloroethane (10 mL) was added, followed by the portionwise addition of ethyl 1*H*-pyrrole-2-carboxylate **3.15**[8] (5.65 g, 41 mmol, 1 equiv) dissolved in 1,2-dichloroethane (10 mL) over 1 h. The solution was refluxed for 15 min, after which it was cooled to r.t. The solution was treated with sodium acetate trihydrate (27.2 g) in water (45 mL), and then refluxed for a further 15 min. The organic layer was separated, and the aqueous layer was extracted with ether (3 x 20 mL). The combined organic fractions were washed with saturated aqueous sodium hydrogen carbonate (3 x 20 mL), dried ($MgSO_4$) and the solvent was removed by evaporation under reduced pressure. The crude material was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:3) to give two compounds. The first to elute was **3.16a** (4.12 g, 61% yield) as an orange solid.

mp 70-73°C (lit.[24] 75°C).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.37 (t, 3H, J = 7.3 Hz, $CH_2C\underline{H}_3$), 4.37 (q, 2H, J = 7.2 Hz, $C\underline{H}_2CH_3$), 6.93 (d, 2H, J = 2.5 Hz, pyrrole H3 and H4), 9.65 (s, 1H, C<u>H</u>O), 9.95 (s(br), 1H, pyrrole N<u>H</u>).

The second to elute was **3.17a** (1.68 g, 25%) as an orange solid.

mp 100-103°C (lit.[24] 104-106°C).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.35 (t, 3H, J = 7.3 Hz, $CH_2C\underline{H}_3$), 4.34 (q, 2H, J = 7.2 Hz, $C\underline{H}_2CH_3$), 7.30 (m, 1H, pyrrole H3), 7.57 (t, 1H, J = 1.5 Hz, pyrrole H5), 9.82 (s, 1H, C<u>H</u>O), 10.39 (s(br), 1H, pyrrole N<u>H</u>).

5-Formyl-1*H*-pyrrole-2-carboxylic acid (3.16b)

Pyrrole ester **3.16a** (4.10 g, 25 mmol, 1 equiv) was hydrolysed with potassium hydroxide (5.61 g, 100 mmol, 4 equiv) by General Procedure F to give **3.16b** (2.03 g, 60%) as an orange solid.

mp 198-204°C (lit.[24] 202-203°C).

^{1}H NMR (acetone-d_6, 300 MHz) δ 7.05 (dd, 1H, J = 1.8, 3.8 Hz, pyrrole H3), 7.15 (dd, 1H, J = 1.8, 3.8 Hz, pyrrole H4), 9.89 (s, 1H, C<u>H</u>O), 11.82 (s(br), 1H, pyrrole N<u>H</u>).

Methyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate (3.18a)

Pyrrole acid **3.16a** (100 mg, 0.72 mmol, 1 equiv) was coupled with methyl L-phenylalaninate hydrochloride (171 mg, 0.79 mmol, 1.1 equiv) by General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:1) to give **3.18a** (152 mg, 71%) as a cream solid.

mp 48-51°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.22 (m, 2H, $C\underline{H}_2Ph$), 3.76 (s, 3H, $CO_2C\underline{H}_3$), 5.06 (m, 1H, NHC<u>H</u>), 6.51 (d, 1H, J = 7.3 Hz, N<u>H</u>CH), 6.61 (dd, 1H, J = 2.7, 4.2 Hz, pyrrole <u>H3</u>), 6.91 (dd, 1H, J = 2.7, 4.2 Hz, pyrrole <u>H4</u>), 7.10 (m, 2H, Ar<u>H</u>), 7.27 (m, 3H, Ar<u>H</u>), 9.61 (s, 1H, C<u>H</u>O), 10.11 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 38.0, 52.6, 53.2, 111.2, 120.0, 127.2, 128.6, 129.3, 131.2, 134.0, 135.5, 159.3, 171.9, 180.3.

FTIR ($CHCl_3$) 1529.4, 1550.7, 1676.0, 1739.7, 3020.3, 3423.4.

HRMS (ES) 301.1188 (M^++H). $C_{16}H_{17}N_2O_4$ requires 301.1188.

Methyl *N*-[(5-hydroxymethyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate (3.13a)

The pyrrole **3.18a** (106 mg, 0.35 mmol, 1 equiv) was reduced with zinc borohydride (5.55 mL of a 0.14 M solution in ether, 0.78 mmol, 2.2 equiv) by General Procedure J. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 4:1) to give **3.13a** (61 mg, 57%) as a tan solid.

mp 105-107°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.97 (s(br), 1H, $CH_2O\underline{H}$), 3.20 (m, 2H, $PhC\underline{H}_2$), 3.74 (s, 3H, $CO_2C\underline{H}_3$), 4.65 (s, 2H, $C\underline{H}_2OH$), 5.00 (m, 1H, NHC<u>H</u>), 6.08 (t, 1H, J = 3.2 Hz, pyrrole <u>H4</u>), 6.37 (d, 1H, J = 7.8 Hz, N<u>H</u>CH), 6.47 (t, 1H, J = 3.2 Hz, pyrrole <u>H3</u>), 7.12 (d, 2H, J = 7.3 Hz, Ar<u>H</u>), 7.27 (m, 3H, Ar<u>H</u>), 10.31 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 37.9 ($PhC\underline{H}_2$), 52.4 ($CO_2C\underline{H}_3$), 53.2 (NH<u>C</u>H), 57.2 ($C\underline{H}_2OH$), 108.1 (pyrrole <u>C4</u>), 110.9 (pyrrole <u>C3</u>), 124.5 (pyrrole <u>C2</u>), 127.1, 128.5, 129.1, 135.7 (Ar<u>C</u>), 136.8 (pyrrole <u>C5</u>), 161.3 (<u>C</u>ONH), 172.4 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1525.6, 1637.5, 1739.7, 3035.7, 3435.0.

HRMS (EI) 302.1266 (M^+). $C_{16}H_{18}N_2O_4$ requires 302.1267.

Micro. Calcd for $C_{16}H_{18}N_2O_4$: C, 63.57; H, 6.00; N, 9.27. Found: C, 63.42; H, 5.90; N, 9.38.

Ethyl 5-(3-phenylpropionyl)-1*H*-pyrrole-2-carboxylate (3.19a)

Pyrrole ester **3.15**[8] (2.91 g, 21 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (7.04 g, 42 mmol, 2 equiv) by General Procedure G. Excess hydrocinnamic acid was removed by dissolving the crude material in ethyl acetate (50 mL) and washing the organic fraction with 1 M aqueous sodium hydroxide solution (4 x 20 mL), after which it was dried ($MgSO_4$). The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 1:4) to give **3.19a** (2.0 g, 35%) as a cream residue.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.36 (t, 3H, J = 7.1 Hz, CH_2CH_3), 3.04 (t, 2H, J = 7.3 Hz, $PhCH_2CH_2$), 3.13 (m, 2H, $PhCH_2CH_2$), 4.35 (q, 2H, J = 7.2 Hz, CH_2CH_3), 6.81 (dd, 1H, J = 2.9, 3.9 Hz, pyrrole H3 or H4), 6.86 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole H3 or H4), 7.18-7.30 (m, 5H, ArH), 9.89 (s(br), 1H, pyrrole NH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2 (CH_2CH_3), 30.2 ($PhCH_2CH_2$), 40.0 ($PhCH_2CH_2$), 61.1 (CH_2CH_3), 115.4, 115.5 (pyrrole C3 and C4), 126.2, 128.3, 128.5, 141.8 (ArC), 127.2 (pyrrole C2), 133.7 (pyrrole C5), 160.3 (CO_2), 190.3 (CH_2CO).

FTIR ($CHCl_3$) 1548.7, 1604.7, 1662.5, 1710.7, 2987.5, 3006.8, 3429.2.

HRMS (EI) 271.1220 (M^+). $C_{16}H_{17}NO_3$ requires 271.1208.

The 4-acyl isomer was not isolated.

Ethyl 5-acetyl-1*H*-pyrrole-2-carboxylate (3.19b)[7]

Pyrrole ester **3.15**[8] (4.0 g, 29 mmol, 1 equiv) was treated with acetyl chloride (4.51 g, 57 mmol, 2 equiv) by General Procedure G. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 1:2) to give **3.19b** (1.93 g, 37%) as a yellow solid.

mp 54-57°C (lit.[7] 60-60.5°C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.36 (t, 3H, J = 7.3 Hz, CH_2CH_3), 2.46 (s, 3H, $COCH_3$), 4.34 (q, 2H, J = 7.2 Hz, CH_2CH_3), 6.83 (t, 1H, J = 3.2 Hz, pyrrole H3 or H4), 6.87 (t, 1H, J = 3.2 Hz, pyrrole H3 or H4), 9.82 (s(br), 1H, pyrrole NH).

The 4-acetyl isomer was not isolated.

5-(3-Phenylpropionyl)-1*H*-pyrrole-2-carboxylic acid (3.20a)

Pyrrole ester **3.19a** (801 mg, 2.95 mmol, 1 equiv) was hydrolysed with potassium hydroxide (663 mg, 11.8 mmol, 4 equiv) by General Procedure H to give **3.20a** (616 mg, 86%) as a brown solid.

mp 181-183°C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 3.11 (t, 2H, J = 7.6 Hz, PhC$\underline{H}_2$CH$_2$), 3.32 (t, 2H, J = 7.6 Hz, PhCH$_2$C$\underline{H}_2$), 6.97 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole H3 or H4), 7.15 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole H3 or H4), 7.29 (m, 1H, Ar$\underline{H}$), 7.39 (m, 4H, Ar$\underline{H}$), 11.23 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR (acetone-d_6, 75 MHz) δ 30.1 (Ph$\underline{C}$H$_2$CH$_2$), 39.9 (PhCH$_2\underline{C}$H$_2$), 115.5, 115.8 (pyrrole C3 and C4), 126.1, 128.5, 128.6, 141.6 (Ar$\underline{C}$), 127.9 (pyrrole C2), 134.8 (pyrrole C5), 161.2 ($\underline{C}$O$_2$H), 190.0 (CH$_2\underline{C}$O).

FTIR (KBr, diffuse refraction method) 1500.5, 1550.7, 1670.2, 1701.1, 2366.5, 2597.9, 3197.8.

HRMS (EI) 243.0900 (M^+). $C_{14}H_{13}NO_3$ requires 243.0895.

5-Acetyl-1*H*-pyrrole-2-carboxylic acid (3.20b)

Pyrrole ester **3.19b** (1.90 g, 10.5 mmol, 1 equiv) was hydrolysed with potassium hydroxide (2.35 g, 42.0 mmol, 4 equiv) by General Procedure H to give **3.20b** (1.50 g, 93%) as a yellow solid.

mp 185-187°C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 2.57 (s, 3H, C$\underline{H}_3$CO), 6.98 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole H3 or H4), 7.11 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole H3 or H4), 11.20 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR (acetone-d_6, 75 MHz) δ 25.5 ($\underline{C}$H$_3$CO), 115.5, 116.2 (pyrrole C3 and C4), 127.9 (pyrrole C2), 135.1 (pyrrole C5), 161.2 ($\underline{C}$O$_2$H), 188.2 (CH$_3\underline{C}$O).

FTIR (KBr, diffuse refraction method) 1546.8, 1670.2, 2360.7, 2511.1, 2584.4, 3124.5, 3436.9.

HRMS (EI) 153.0426 (M^+). $C_7H_7NO_3$ requires 153.0426.

Micro. Calcd for $C_7H_7NO_3$: C, 54.90; H, 4.61; N, 9.15. Found: C, 54.93; H, 4.55; N, 9.07.

Methyl *N*-{[5-(3-phenylpropionyl)-1*H*-pyrrol-2-yl]carbonyl}-L-phenylalaninate (3.21a)

Pyrrole acid **3.20a** (150 mg, 0.62 mmol, 1 equiv) was coupled with methyl L-phenylalaninate hydrochloride (146 mg, 0.68 mmol, 1.1 equiv) by modified General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:3) to give **3.21a** (175 mg, 70%) as a brown residue.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.03 (m, 2H, $PhC\underline{H}_2CH_2$), 3.14 (m, 2H, $PhCH_2C\underline{H}_2$), 3.21 (m, 2H, $C\underline{H}_2Ph$), 3.75 (s, 3H, $CO_2C\underline{H}_3$), 5.05 (m, 1H, NHC<u>H</u>), 6.46 (m, 2H, pyrrole <u>H3</u> and N<u>H</u>CH), 6.78 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole <u>H4</u>), 7.10 (m, 2H, Ar<u>H</u>), 7.17-7.30 (m, 8H, Ar<u>H</u>), 10.00 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 30.3 ($Ph\underline{C}H_2CH_2$), 37.9 ($CH\underline{C}H_2Ph$), 40.0 ($PhCH_2\underline{C}H_2$), 52.5 ($CO_2\underline{C}H_3$), 53.1 (NH<u>C</u>H), 110.4 (pyrrole <u>C3</u>), 115.5 (pyrrole <u>C4</u>), 126.2, 127.2, 128.3, 128.5, 128.6, 129.3, 135.6, 140.9 (Ar<u>C</u>), 129.7 (pyrrole <u>C2</u>), 133.3 (pyrrole <u>C5</u>), 159.4 (<u>C</u>ONH), 171.9 (<u>C</u>O_2Me), 190.0 ($CH_2\underline{C}O$).

FTIR ($CHCl_3$) 1529.4, 1548.7, 1668.3, 1741.6, 3008.7, 3425.3.

HRMS (EI) 404.1745 (M^+). $C_{24}H_{24}N_2O_3$ requires 404.1736.

Micro. Calcd for $C_{24}H_{24}N_2O_4$: C, 71.27; H, 5.98; N, 6.93. Found: C, 71.37; H, 6.15; N, 7.10.

Methyl *N*-[(5-acetyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate (3.21b)

Pyrrole acid **3.20b** (200 mg, 1.31 mmol, 1 equiv) was coupled with methyl L-phenylalaninate hydrochloride (310 mg, 1.44 mmol, 1.1 equiv) by modified General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:1) to give **3.21b** (375 mg, 91%) as a cream solid. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **3.21b** dissolved in ethyl acetate.

mp 103-105°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.42 (s, 3H, $C\underline{H}_3CO$), 3.20 (m, 2H, $C\underline{H}_2Ph$), 3.75 (s, 3H, $CO_2C\underline{H}_3$), 5.06 (m, 1H, NHC<u>H</u>), 6.52 (dd, 1H, J = 2.9, 3.9 Hz, pyrrole <u>H3</u>), 6.67 (d, 1H, J = 7.8 Hz, N<u>H</u>CH), 6.88 (dd, 1H, J = 2.7, 4.2 Hz, pyrrole <u>H4</u>), 7.10 (m, 2H, Ar<u>H</u>), 7.25 (m, 3H, Ar<u>H</u>), 10.11 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.8 ($\underline{C}H_3CO$), 37.9 ($\underline{C}H_2Ph$), 52.5 ($CO_2\underline{C}H_3$), 53.2 (NH$\underline{C}$H), 110.9 (pyrrole $\underline{C}$3), 116.3 (pyrrole $\underline{C}$4), 127.2, 128.6, 129.2, 135.7 (Ar$\underline{C}$), 130.0 (pyrrole $\underline{C}$2), 133.6 (pyrrole $\underline{C}$5), 159.4 ($\underline{C}$ONH), 172.1 ($\underline{C}O_2$), 188.6 ($CH_3\underline{C}O$).

FTIR (KBr, diffuse refraction method) 1546.8, 1654.8, 1741.6, 2956.7, 3031.9, 3112.9, 3352.1.

HRMS (EI) 314.1271 (M^+). $C_{17}H_{18}N_2O_4$ requires 314.1267.

Micro. Calcd for $C_{17}H_{18}N_2O_4$: C, 64.96; H, 5.77; N, 8.91. Found: C, 65.13; H, 5.84; N, 8.77.

Methyl (*RS*)-*N*-{[5-(1-hydroxy-3-phenylpropyl)-1*H*-pyrrol-2-yl]carbonyl}-L-phenylalaninate (3.14a)

The pyrrole **3.21a** (112 mg, 0.28 mmol, 1 equiv) was reduced with zinc borohydride (4.35 mL of a 0.14 M solution in ether, 0.61 mmol, 2.2 equiv) by General Procedure J. The crude material was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 1:1) to give three compounds. The first fraction to elute contained a mixture of starting material **3.21a** and the deoxygenated byproduct methyl *N*-[(5-(3-phenylpropyl)-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate **3.14c** (41 mg). Spectral data for **3.14c** (from mixture).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.95 (m, 2H, $C\underline{H}_2$), 2.63 (m, 4H, 2 x $C\underline{H}_2$), 3.18 (m, 2H, $C\underline{H}_2Ph$), 3.71 (s, 3H, $CO_2C\underline{H}_3$), 5.03 (m, 1H, NHC$\underline{H}$), 5.93 (m, 1H, pyrrole $\underline{H}$4), 6.23 (d, 1H, J = 7.8 Hz, N$\underline{H}$CH), 6.45 (m, 1H, pyrrole $\underline{H}$3), 7.11-7.29 (m, 10H, Ar$\underline{H}$), 9.35 (s(br), 1H, pyrrole N$\underline{H}$).

HRMS (EI) 390.1953 (M^+). $C_{24}H_{26}N_2O_3$ requires 390.1943.

The second fraction to elute contained **3.14a** (24 mg, 21%, 1:1 mixture of diastereoisomers) as a light yellow oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.13 (m, 2H, $PhCH_2C\underline{H}_2$), 2.69-2.82 (m, 2H, $PhC\underline{H}_2CH_2$), 3.20 (m, 2H, $CHC\underline{H}_2Ph$), 3.71 and 3.72 (s, each 3H, $CO_2C\underline{H}_3$, isomer A and B), 4.75 (m, 1H, C$\underline{H}$OH), 5.02 (m, 1H, NHC$\underline{H}$), 6.02 (m, 1H, pyrrole $\underline{H}$4), 6.42 (d, 1H, *J* = 7.3 Hz, N$\underline{H}$CH), 6.48 (m, 1H, pyrrole $\underline{H}$3), 7.11-7.29 (m, 10H, Ar$\underline{H}$), 10.67 and 10.70 (s(br), each 1H, pyrrole N$\underline{H}$, isomer A and B).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 31.8 (Ph$\underline{C}H_2CH_2$), 38.2 ($CH\underline{C}H_2Ph$), 38.6 and 38.7 ($PhCH_2\underline{C}H_2$, isomers A and B), 52.4 ($CO_2\underline{C}H_3$), 53.1 (NH$\underline{C}$H), 67.1 and 67.1 ($\underline{C}$HOH, isomers A and B), 106.4 and 106.5 (pyrrole $\underline{C}$4, isomers A and B), 110.7 (pyrrole $\underline{C}$3), 124.0 (pyrrole $\underline{C}$2), 125.9, 127.2, 128.4, 128.5, 128.6, 129.3, 135.6, 141.6 (Ar$\underline{C}$), 140.1 (pyrrole $\underline{C}$5), 161.1 ($\underline{C}$ONH), 172.0 ($\underline{C}O_2$Me).

FTIR ($CHCl_3$) 1521.7, 1602.7, 1641.3, 1741.6, 3012.6, 3030.0, 3436.9.

HRMS (EI) 388.1800 (M^+-H_2O). $C_{24}H_{24}N_2O_3$ requires 388.1787.

Methyl (*RS*)-*N*-{[5-(1-hydroxyethyl)-1*H*-pyrrol-2-yl]carbonyl}-L-phenylalaninate (3.14b)

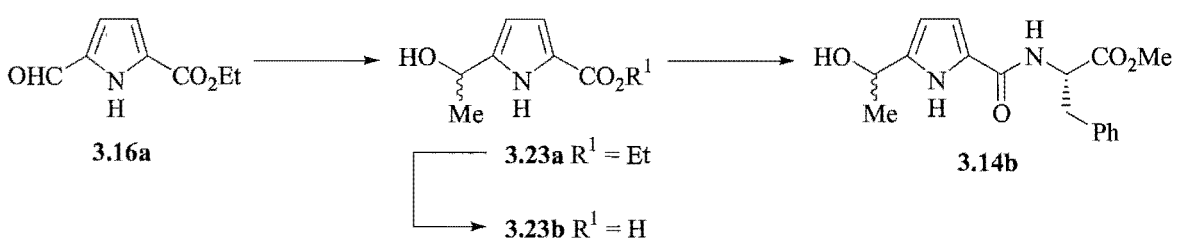
Method A

The pyrrole **3.21b** (150 mg, 0.48 mmol, 1 equiv) was reduced with lithium borohydride (21 mg, 0.95 mmol, 2 equiv) according to General Procedure K. The crude product was partially purified flash chromatography on silica (ethyl acetate/petroleum ether, 4:1) to give **3.14b** (70 mg, 46%) as a slightly impure light green residue by ^{1}H NMR spectroscopy.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.53 (m, 3H, $C\underline{H}_3$CHOH), 3.20 (m, 2H, $C\underline{H}_2$Ph), 3.74 (s, 3H, $CO_2C\underline{H}_3$), 4.90 (m, 1H, C$\underline{H}$OH), 5.03 (dd, 1H, J = 5.9, 12.2 Hz, NHC$\underline{H}$), 6.00 (s, 1H, pyrrole $\underline{H}$4), 6.48 (t, 1H, J = 2.7 Hz, pyrrole $\underline{H}$3), 6.68 (m, 1H, N$\underline{H}$CH), 7.15 (d, 2H, J = 6.8 Hz, Ar$\underline{H}$), 7.26 (m, 3H, Ar$\underline{H}$), 10.71 and 10.73 (s(br), each 1H, pyrrole N$\underline{H}$, isomers A and B).

No further analytical data was obtained due to product degradation.

6.3.2 Attempted preparation of the C5-(hydroxyalkyl)pyrrole 3.14b



Ethyl (*RS*)-5-(1-hydroxyethyl)-1*H*-pyrrole-2-carboxylate (3.23a)

A stirred solution of the pyrrole ester **3.16a** (400 mg, 2.39 mmol, 1 equiv) in dry THF (40 mL) under an inert atmosphere was cooled to –23°C (dry ice/carbon tetrachloride). Methyllithium (1.50 mL of a 1.6 M solution in ether, 2.39 mmol, 1 equiv) was added over 30 min, and the solution was stirred at –23°C for 2.5 h. After this, a further 1.50 mL of the methyllithium solution was added over 30 min, and the solution was again stirred at –23°C for 2.5 h. The resultant solution was poured onto an ether/ice bath, and once the ice had melted the layers were separated. The organic layer was washed with aqueous saturated brine (25 mL), water (2 x 25 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure to yield **3.23a** (418 mg, 95%) as a brown oil. (NOTE: In previous experiments, the title product was isolated from the crude material by flash chromatography on silica, using ethyl acetate/petroleum ether, 3:1, as the eluant.)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.34 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$), 1.55 (d, 3H, J = 6.8 Hz, $C\underline{H}_3CHOH$), 3.08 (s(br), 1H, $CHO\underline{H}$), 4.29 (q, 2H, J = 7.2 Hz, $C\underline{H}_2CH_3$), 4.96 (q, 1H, J = 6.5 Hz, $CH_3C\underline{H}OH$), 6.05 (t, 1H, J = 3.2 Hz, pyrrole H4), 6.83 (t, 1H, J = 3.2 Hz, pyrrole H3), 9.77 (s(br), 1H pyrrole N$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.4 ($CH_2\underline{C}H_3$), 23.2 ($\underline{C}H_3CHOH$), 60.4 ($\underline{C}H_2CH_3$), 64.0 ($CH_3\underline{C}HOH$), 106.3 (pyrrole C4), 115.9 (pyrrole C3), 121.8 (pyrrole C2), 141.5 (pyrrole C5), 161.9 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1693.4, 2983.7, 3020.3, 3446.6.

HRMS (EI) 183.0897 (M^+). $C_9H_{13}NO_3$ requires 183.0895.

Micro. Calcd for $C_9H_{13}NO_3$: C, 59.00; H, 7.15; N, 7.65. Found: C, 58.60; H, 7.22; N, 7.60.

(*RS*)-5-(1-Hydroxyethyl)-1*H*-pyrrole-2-carboxylic acid (3.23b)

A stirred solution of the pyrrole ester **3.23a** (575 mg, 3.13 mmol, 1 equiv) in 1:1 THF/water (10 mL) under an inert atmosphere was warmed to 40-50°C. Sodium hydroxide (377 mg, 9.42 mmol, 3 equiv) was added, and the progress of the reaction was monitored by TLC (ethyl acetate/petroleum ether, 1:1). After 24 h, the solution was cooled to r.t., washed with ether (10 mL), cooled to 0°C and acidified to pH ~4 by the dropwise addition of 1M aqueous hydrochloric acid. The resulting solution was extracted with ether

(3 x 15 mL), the combined ethereal extracts were washed with aqueous saturated brine (15 mL), dried (MgSO4), and the solvent was removed by evaporation under reduced pressure to give **3.23b** (85 mg, 17%) as a purple solid. This product was used without further purification.

mp 110-115°C.

^{1}H NMR (acetone-d_6, 300 MHz) δ 1.59 (d, 3H, J = 6.3 Hz, C$\underline{H}_3$CHOH), 4.43 (s(br), 1H, CHO$\underline{H}$), 5.02 (m, 1H, CH$_3$C$\underline{H}$OH), 6.20 (t, 1H, J = 3.2 Hz, pyrrole $\underline{H4}$), 6.87 (t, 1H, J = 3.2 Hz, pyrrole $\underline{H3}$), 10.47 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR (acetone-d_6, 75 MHz) δ 23.2 (C$\underline{H}_3$CHOH), 63.3 (CH$_3$C$\underline{H}$OH), 105.9 (pyrrole $\underline{C4}$), 115.6 (pyrrole $\underline{C3}$), 121.8 (pyrrole $\underline{C5}$), 143.2 (pyrrole $\underline{C2}$), 161.8 ($\underline{C}$O$_2$H).

FTIR (KBr, diffuse refraction method) 1651.0, 3230.5, 3419.6.

HRMS (EI) 155.0579 (M$^+$). $C_7H_9NO_3$ requires 155.0582.

Methyl (*RS*)-*N*-{[5-(1-hydroxyethyl)-1*H*-pyrrol-2-yl]carbonyl}-L-phenylalaninate (3.14b)

Method B

Pyrrole acid **3.23b** (20 mg, 0.13 mmol, 1 equiv) was coupled with methyl L-phenylalaninate hydrochloride (31 mg, 0.14 mmol, 1.1 equiv) by General Procedure I, except in the workup whereby 0.1 M hydrochloric acid was used instead of the 3 M hydrochloric acid. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 4:1) to give **3.14b** (22 mg, 54%) as a colourless oil.

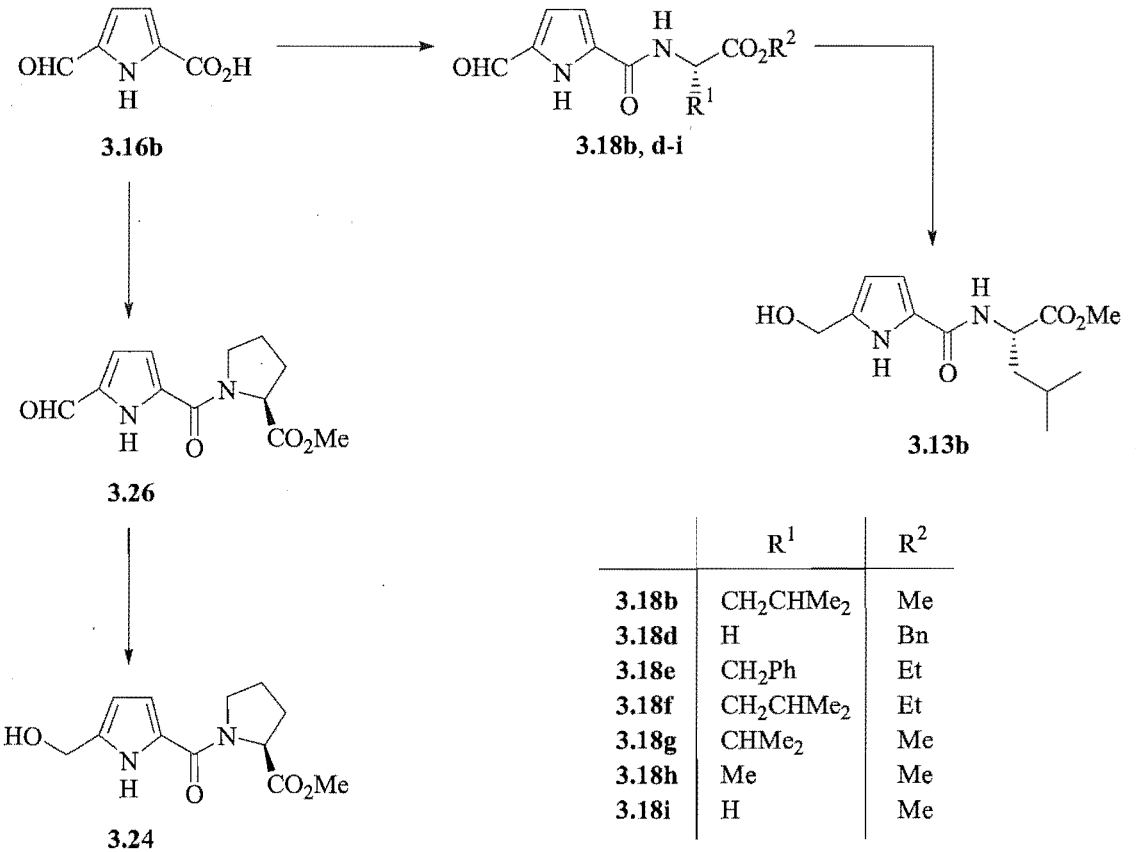
^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.53 (m, 3H, C$\underline{H}_3$CHOH), 3.20 (m, 2H, C$\underline{H}_2$Ph), 3.73 (s, 3H, CO$_2$C$\underline{H}_3$), 4.89-5.05 (m, 2H, CH$_3$C$\underline{H}$OH plus NHC$\underline{H}$), 6.00 (d, 1H, J = 2.0 Hz, pyrrole $\underline{H4}$), 6.48 (m, 1H, pyrrole $\underline{H3}$) 6.55 (s(br), 1H, N$\underline{H}$CH), 7.13 (d, 2H, J = 7.3 Hz, Ar$\underline{H}$), 7.25 (m, 3H, Ar$\underline{H}$), 10.65 (s(br), 1H, pyrrole N$\underline{H}$).

FTIR ($CHCl_3$) 1521.7, 1645.2, 1739.7, 3022.2, 3435.0.

HRMS (EI) 316.1423 (M$^+$). $C_{17}H_{20}N_2O_4$ requires 316.1423.

No further analytical data was obtained due to product degradation.

6.3.3 Preparation of the 5-formylpyrroles 3.18d-i, and the 5-(hydroxymethyl)-pyrroles 3.13b and 3.24



	R^1	R^2
3.18b	CH_2CHMe_2	Me
3.18d	H	Bn
3.18e	CH_2Ph	Et
3.18f	CH_2CHMe_2	Et
3.18g	$CHMe_2$	Me
3.18h	Me	Me
3.18i	H	Me

Methyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-leucinate (3.18b)

The pyrrole acid **3.16b** (100 mg, 0.72 mmol, 1 equiv) was coupled with methyl L-leucinate hydrochloride (144 mg, 0.79 mmol, 1.1 equiv) by General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:1) to give **3.18b** (116 mg, 61%) as a cream solid.

mp 139-142°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.96 (m, 6H, $CH(C\underline{H}_3)_2$), 1.61-1.77 (m, 3H, $C\underline{H}_2C\underline{H}(CH_3)_2$), 3.77 (s, 3H, $CO_2C\underline{H}_3$), 4.83 (m, 1H, NHC<u>H</u>), 6.62 (d, 1H, J = 8.3 Hz, N<u>H</u>CH), 6.68 (d, 1H, J = 2.4 Hz, pyrrole <u>H3</u>) 6.93 (d, 1H, J = 2.9 Hz, pyrrole <u>H4</u>), 9.62 (s, 1H, C<u>H</u>O), 10.18 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 21.9, 22.8, 24.9, 41.7, 50.9, 52.6, 111.0, 120.0, 131.2, 133.9, 159.6, 173.7, 180.2.

FTIR ($CHCl_3$) 1529.4, 1550.7, 1676.0, 1739.7, 2960.5, 3018.4, 3425.3.

HRMS (EI) 266.1266 (M^+). $C_{13}H_{18}N_2O_4$ requires 266.1267.

Benzyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]glycinate (3.18d)

The pyrrole acid **3.16b** (120 mg, 0.86 mmol, 1 equiv) was coupled with benzyl glycinate hydrochloride (191 mg, 0.95 mmol, 1.1 equiv) by General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 3:2) to give **3.18d** (138 mg, 56%) as an orange solid.

mp 137-139°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.26 (d, 2H, J = 4.9 Hz, NHC$\underline{H}_2$), 5.22 (s, 2H, C$\underline{H}_2$Ph), 6.69 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole $\underline{H}$3), 6.73 (s(br), 1H, N$\underline{H}$CH$_2$), 6.93 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole $\underline{H}$4), 7.36 (m, 5H, Ar$\underline{H}$), 9.62 (s, 1H, C$\underline{H}$O), 10.30 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 41.5 (NH$\underline{C}$H$_2$), 67.5 ($\underline{C}$H$_2$Ph), 111.3 (pyrrole $\underline{C}$3), 120.0 (pyrrole $\underline{C}$4), 128.4, 128.6, 128.7, 131.0 (Ar$\underline{C}$), 134.0 (pyrrole $\underline{C}$2), 134.9 (pyrrole $\underline{C}$5), 160.0 ($\underline{C}$ONH), 169.8 ($\underline{C}$O$_2$), 180.3 ($\underline{C}$HO).

FTIR ($CHCl_3$) 1533.3, 1552.6, 1678.0, 1743.5, 3031.9, 3425.3.

HRMS (EI) 286.0952 (M^+). $C_{15}H_{14}N_2O_4$ requires 286.0954.

Micro. Calcd for $C_{15}H_{14}N_2O_4$: C, 62.93; H, 4.93; N, 9.79. Found: C, 62.90; H, 4.86; N, 10.09.

Ethyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate (3.18e)

The pyrrole acid **3.16b** (80 mg, 0.58 mmol, 1 equiv) was coupled with ethyl L-phenylalaninate hydrochloride (145 mg, 0.63 mmol, 1.1 equiv) by modified General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 1:1) to give **3.18e** (104 mg, 58%) as a cream solid.

mp 42-45°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.28 (t, 3H, J = 7.1 Hz, CH$_2$C$\underline{H}_3$), 3.24 (m, 2H, C$\underline{H}_2$Ph), 4.23 (q, 2H, J = 7.0 Hz, C$\underline{H}_2$CH$_3$), 5.09 (m, 1H, NHC$\underline{H}$), 6.63 (d, 1H, J = 3.9 Hz, pyrrole $\underline{H}$3), 6.81 (d, 1H, J = 7.8 Hz, N$\underline{H}$CH), 6.93 (d, 1H, J = 4.4 Hz, pyrrole $\underline{H}$4), 7.16 (m, 2H, Ar$\underline{H}$), 7.27 (m, 3H, Ar$\underline{H}$), 9.61 (s, 1H, C$\underline{H}$O), 10.50 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1 ($CH_2\underline{C}H_3$), 38.0 ($\underline{C}H_2Ph$), 53.3 (NH$\underline{C}$H), 61.8 ($\underline{C}H_2CH_3$), 111.2 (pyrrole $\underline{C}$3), 119.9 (pyrrole $\underline{C}$4), 127.2, 128.5, 129.3, 135.6 (Ar$\underline{C}$), 131.3 (pyrrole $\underline{C}$2), 134.0 (pyrrole $\underline{C}$5), 159.3 ($\underline{C}$ONH), 171.5 ($\underline{C}O_2$), 180.3 ($\underline{C}$HO).

FTIR ($CHCl_3$) 1529.4, 1550.7, 1676.0, 1733.9, 2987.5, 3423.4.

HRMS (EI) 314.1269 (M^+). $C_{17}H_{18}N_2O_4$ requires 314.1267.

Micro. Calcd for $C_{17}H_{18}N_2O_4$: C, 64.96; H, 5.77; N, 8.91. Found: C, 64.68; H, 5.98; N, 9.20.

Ethyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-leucinate (3.18f)

The pyrrole acid **3.16b** (80 mg, 0.58 mmol, 1 equiv) was coupled with ethyl L-leucinate hydrochloride (124 mg, 0.63 mmol, 1.1 equiv) by General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 1:1) to give **3.18f** (61 mg, 38%) as an orange oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.95 (m, 6H, CH($C\underline{H}_3$)$_2$), 1.29 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$), 1.61-1.74 (m, 3H, $C\underline{H}_2C\underline{H}$), 4.22 (q, 2H, J = 7.2 Hz, $C\underline{H}_2CH_3$), 4.81 (m, 1H, NHC$\underline{H}$), 6.69 (d, 1H, J = 3.9 Hz, pyrrole $\underline{H}$3), 6.90 (d, 1H, J = 3.9 Hz, pyrrole $\underline{H}$4), 6.98 (d, 1H, J = 8.3 Hz, N$\underline{H}$CH), 9.59 (s, 1H, C$\underline{H}$O), 10.36 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.0 ($CH_2\underline{C}H_3$), 21.5, 22.7 (CH($\underline{C}H_3$)$_2$), 24.8, 40.9 ($\underline{C}H_2\underline{C}H$), 50.9 (NH$\underline{C}$H), 61.6 ($\underline{C}H_2CH_3$), 111.7 (pyrrole $\underline{C}$3), 119.5 (pyrrole $\underline{C}$4), 131.4 (pyrrole $\underline{C}$2), 133.9 (pyrrole $\underline{C}$5), 159.9 ($\underline{C}$ONH), 173.9 ($\underline{C}O_2$), 180.4 ($\underline{C}$HO).

FTIR ($CHCl_3$) 1529.4, 1550.7, 1676.0, 1732.0, 2964.4, 3010.7, 3033.8, 3425.3.

HRMS (EI) 280.1419 (M^+). $C_{14}H_{20}N_2O_4$ requires 280.1423.

Micro. Calcd for $C_{14}H_{20}N_2O_4$: C, 59.99; H, 7.19; N, 9.99. Found: C, 60.06; H, 7.27; N, 10.01.

Methyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-valinate (3.18g)

The pyrrole acid **3.16b** (120 mg, 0.86 mmol, 1 equiv) was coupled with methyl L-valinate hydrochloride (159 mg, 0.95 mmol, 1.1 equiv) by modified General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/dichloromethane, 1:1) to give **3.18g** (115 mg, 53%) as a cream solid.

mp 146-148°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.97 (m, 6H, $CH(C\underline{H}_3)_2$), 2.25 (m, 1H, $C\underline{H}(CH_3)_2$), 3.77 (s, 3H, $CO_2C\underline{H}_3$), 4.70 (m, 1H, NHC<u>H</u>), 6.74 (d, 1H, J = 4.6 Hz, pyrrole <u>H3</u>), 6.85 (d, 1H, J = 8.3 Hz, N<u>H</u>CH), 6.95 (d, 1H, J = 4.4 Hz, pyrrole <u>H4</u>), 9.62 (s, 1H, C<u>H</u>O), 10.54 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.9, 18.9 ($CH(\underline{C}H_3)_2$), 31.6 ($\underline{C}H(CH_3)_2$), 52.4 ($CO_2\underline{C}H_3$), 57.2 (NH<u>C</u>H), 111.2 (pyrrole <u>C3</u>), 120.1 (pyrrole <u>C4</u>), 131.4 (pyrrole <u>C2</u>), 133.9 (pyrrole <u>C5</u>), 159.7 (<u>C</u>ONH), 172.5 ($\underline{C}O_2$), 180.3 (<u>C</u>HO).

FTIR ($CHCl_3$) 1529.4, 1548.7, 1678.0, 1735.8, 2968.2, 3033.8, 3425.3.

HRMS (EI) 252.1113 (M^+). $C_{12}H_{16}N_2O_4$ requires 252.1110.

Micro. Calcd for $C_{12}H_{14}N_2O_4$: C, 57.13; H, 6.39; N, 11.10. Found: C, 57.32; H, 6.46; N, 11.16.

Methyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-alaninate (3.18h)

The pyrrole acid **3.16b** (75 mg, 0.54 mmol, 1 equiv) was coupled with methyl L-alaninate hydrochloride (83 mg, 0.59 mmol, 1.1 equiv) by General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 3:1) to give **3.18h** (52 mg, 43%) as a cream solid.

mp 123-126°C.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.51 (d, 3H, J = 6.8 Hz, $CHC\underline{H}_3$), 3.79 (s, 3H, $CO_2C\underline{H}_3$), 4.79 (m, 1H, NHC<u>H</u>), 6.68 (m, 1H, pyrrole <u>H3</u>), 6.77 (m, 1H, N<u>H</u>CH), 6.94 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole <u>H4</u>), 9.62 (s, 1H, C<u>H</u>O), 10.27 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 18.4, 48.2, 52.7, 111.4, 120.0, 131.4, 134.0, 159.5, 173.6, 180.4.

FTIR ($CHCl_3$) 1529.4, 1550.7, 1676.0, 1739.7, 3008.7, 3035.7, 3423.4.

HRMS (EI) 224.0797 (M^+). $C_{10}H_{12}N_2O_4$ requires 224.0797.

Methyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]glycinate (3.18i)

The pyrrole acid **3.16b** (10 mg, 0.72 mmol, 1 equiv) was coupled with methyl glycinate hydrochloride (99 mg, 0.79 mmol, 1.1 equiv) by General Procedure M. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 3:1) to give

3.18i (66 mg, 44%) as a cream solid. An analytical sample was obtained by recrystallisation from ethyl acetate/petroleum ether to give a white crystalline solid.

mp 209-211°C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 3.81 (s, 3H, CO_2CH_3), 4.24 (d, 2H, J = 5.9 Hz, $NHCH_2$), 7.07 (m, 1H, pyrrole H3 or H4), 7.13 (m, 1H, pyrrole H3 or H4), 8.26 (s(br), 1H, $NHCH_2$), 9.85 (s, 1H, CHO), 11.53 (s(br), 1H, pyrrole NH).

^{13}C NMR (acetone-d_6 + 3 drops DMSO-d_6, 75 MHz) δ 40.7 ($NHCH_2$), 51.4 (CO_2CH_3), 112.8 (pyrrole C3), 117.3 (pyrrole C4), 132.2 (pyrrole C2), 134.7 (pyrrole C5), 160.1 (CONH), 170.3 (CO_2), 180.8 (CHO).

FTIR (KBr, diffuse refraction method) 1560.3, 1641.3, 1685.7, 1724.2, 3136.0, 3211.3, 3373.3.

HRMS (EI) 210.0646 (M^+). $C_9H_{10}N_2O_4$ requires 210.0641.

Methyl *N*-[(5-hydroxymethyl-1*H*-pyrrol-2-yl)carbonyl]-L-leucinate (3.13b)

Method A

The pyrrole **3.18b** (116 mg, 0.44 mmol, 1 equiv) was reduced with zinc borohydride (6.85 mL of a 0.14 M solution in ether, 0.96 mmol, 2.2 equiv) by General Procedure J. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 4:1) to give **3.18b** (49 mg, 42%) as a cream solid.

mp 92-96°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.94 (m, 6H, $CH(CH_3)_2$), 1.63-1.75 (m, 3H, CH_2CH), 3.76 (s, 3H, CO_2CH_3), 4.60 (d, 2H, J = 2.9 Hz, CH_2OH), 4.76 (m, 1H, NHCH), 6.04 (dd, 1H, J = 2.4, 3.4 Hz, pyrrole H4), 6.57 (dd, 1H, J = 2.4, 3.4 Hz, pyrrole H3), 6.62 (d, 1H, J = 8.8 Hz, NHCH), 10.51 (s(br), 1H, pyrrole NH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 21.6, 22.8 ($CH(CH_3)_2$), 24.8 (CH_2CH), 41.0 (CH_2CH), 50.7 (NHCH), 52.4 (CO_2CH_3), 57.3 (CH_2OH), 107.9 (pyrrole C4), 110.9 (pyrrole C3), 124.6 (pyrrole C2), 136.8 (pyrrole C5), 161.7 (CONH), 174.5 (CO_2).

FTIR ($CHCl_3$) 1525.6, 1641.3, 1739.7, 2958.6, 3438.8.

HRMS (EI) 268.1418 (M^+). $C_{13}H_{20}N_2O_4$ requires 268.1423.

Micro. Calcd for $C_{13}H_{20}N_2O_4$: C, 58.19; H, 7.51; N, 10.44. Found: C, 58.35; H, 7.37; N, 10.36.

Method B

The pyrrole **3.18b** (35 mg, 0.13 mmol, 1 equiv) was reduced with lithium borohydride (6 mg, 0.26 mmol, 2 equiv) by modified General Procedure K. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 4:1) to give **3.13b** (23 mg, 66%) as an off-white solid.

mp 94-97°C.

^{1}H NMR data was identical for that recorded above.

Micro. Calcd for $C_{13}H_{20}N_2O_4$: C, 58.19; H, 7.51; N, 10.44. Found: C, 58.42; H, 7.29; N, 10.51.

Methyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-prolinate (3.26)

The pyrrole acid **3.16b** (100 mg, 0.72 mmol, 1 equiv) was coupled with methyl L-prolinate hydrochloride (131 mg, 0.79 mmol, 1.1 equiv) by modified General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:1 then 4:1) to give **3.26** (133 mg, 74%) as a white solid.

mp decomp. >40°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.01-2.27 (m, 4H, $NCH_2C\underline{H}_2C\underline{H}_2CH$), 3.73 (s, 3H, $CO_2C\underline{H}_3$), 3.86 (m, 1H, $NC\underline{H}_{2a}$), 3.97 (m, 1H, $NC\underline{H}_{2b}$), 4.70 (m, 1H, $NC\underline{H}$), 6.69 (m, 1H, pyrrole H3), 6.92 (m, 1H, pyrrole H4), 9.63 (s, 1H, C<u>H</u>O), 10.38 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.3, 28.6 ($NCH_2\underline{C}H_2\underline{C}H_2CH$), 48.5 ($N\underline{C}H_2$), 52.3 ($CO_2\underline{C}H_3$), 60.2 ($N\underline{C}H$), 113.2 (pyrrole C3), 119.3 (pyrrole C4), 130.7 (pyrrole C2), 133.3 (pyrrole C5), 159.5 (<u>C</u>ONH), 172.3 ($\underline{C}O_2$), 180.1 (<u>C</u>HO).

FTIR ($CHCl_3$) 1544.9, 1616.2, 1672.2, 1745.5, 3419.6.

HRMS (EI) 250.0952 (M^+). $C_{12}H_{14}N_2O_4$ requires 250.0954.

Micro. Calcd for $C_{12}H_{14}N_2O_4$: C, 57.59; H, 5.64; N, 11.19. Found: C, 57.63; H, 5.73; N, 11.21.

Methyl *N*-[(5-hydroxymethyl-1*H*-pyrrol-2-yl)carbonyl]-L-prolinate (3.24)

The pyrrole **3.26** (120 mg, 0.48 mmol, 1 equiv) was reduced with zinc borohydride (7.54 mL of a 0.14 M solution in ether, 1.05 mmol, 2.2 equiv) by General Procedure J. The

crude product was purified by flash chromatography on silica (ethyl acetate/acetone, 10:1) to give **3.24** (29 mg, 24%) as an orange oil.

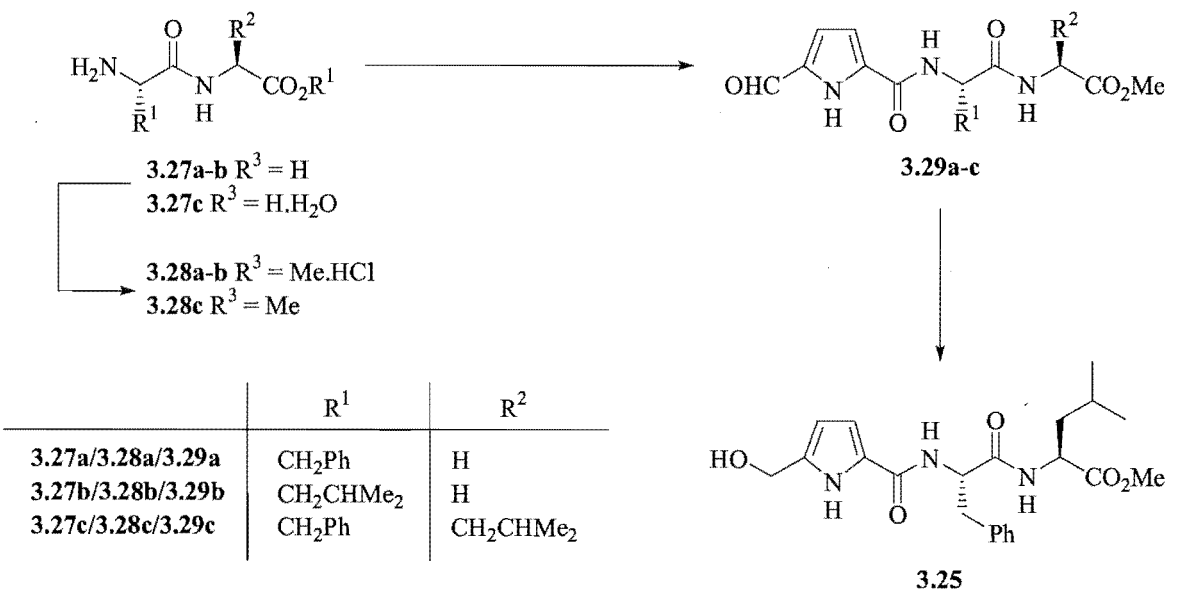
^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.03-2.24 (m, 4H, $NCH_2C\underline{H}_2C\underline{H}_2CH$), 3.44 (s(br), 1H, O<u>H</u>), 3.73 (s, 3H, $CO_2C\underline{H}_3$), 3.85 (m, 1H, $NC\underline{H}_{2a}$), 3.95 (m, 1H, $NC\underline{H}_{2b}$), 4.61 (s, 2H, $C\underline{H}_2OH$), 4.73 (m, 1H, NC<u>H</u>), 6.13 (s, 1H, pyrrole <u>H4</u>), 6.60 (s, 1H, pyrrole <u>H3</u>), 10.75 (s(br), 1H, pyrrole N<u>H</u>).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.2, 28.8 ($NCH_2\underline{C}H_2\underline{C}H_2CH$), 48.8 ($N\underline{C}H_2$), 52.4 ($CO_2\underline{C}H_3$), 57.4 ($\underline{C}H_2OH$), 60.2 (N<u>C</u>H), 108.2 (pyrrole <u>C4</u>), 113.8 (pyrrole <u>C3</u>), 124.6 (pyrrole <u>C2</u>), 136.9 (pyrrole <u>C5</u>), 161.3 (<u>C</u>ON), 172.8 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1596.9, 1743.5, 3010.7, 3436.9, 3691.5.

HRMS (EI) 252.1119 (M^+). $C_{12}H_{16}N_2O_4$ requires 252.1110.

6.3.4 Preparation of the 5-formylpyrroles 3.29a-b, and the 5-(hydroxymethyl)-pyrrole 3.25



	R^1	R^2
3.27a/3.28a/3.29a	CH_2Ph	H
3.27b/3.28b/3.29b	CH_2CHMe_2	H
3.27c/3.28c/3.29c	CH_2Ph	CH_2CHMe_2

Methyl *N*-(L-phenylalanyl)glycinate hydrochloride (3.28a)

N-(L-Phenylalanyl)glycine **3.27a** (180 mg, 0.80 mmol, 1 equiv) was esterified with methanol by General Procedure O. The crude product was purified by flash

chromatography on silica (1:1 ethyl acetate/methanol, visualising agent ninhydrin) to give **3.28a** (197 mg, 89%) as a white solid.

mp 135-138°C (lit.[25] 136-138°C).

^{1}H NMR (CD_3OD, 500 MHz) δ 3.05 (dd, 1H, J = 8.3, 14.2 Hz, C$\underline{H}_{2a}$Ph), 3.26 (dd, 1H, J = 5.9, 14.2 Hz, C$\underline{H}_{2b}$Ph), 3.73 (s, 3H, $CO_2C\underline{H}_3$), 3.98 (s, 2H, NHC$\underline{H}_2$), 4.13 (m, 1H, $H_2NC\underline{H}$), 7.34 (m, 5H, Ar$\underline{H}$).

Methyl *N*-(L-leucyl)glycinate hydrochloride (3.28b)

N-(L-Leucyl)glycine **3.27b** (500 mg, 2.66 mmol, 1 equiv) was esterified with methanol by General Procedure O. The crude product was purified by flash chromatography on silica (ethyl acetate/methanol, 2:1, visualising agent ninhydrin) to give **3.28b** in a quantitative yield as a hygroscopic white solid.

^{1}H NMR (CD_3OD, 500 MHz) δ 1.01 (m, 6H, CH(C$\underline{H}_3$)$_2$), 1.65-1.78 (m, 3H, C$\underline{H}_2$C$\underline{H}$), 3.73 (s, 3H, $CO_2C\underline{H}_3$), 3.91 (m, 1H, $H_2NC\underline{H}$), 4.01 (AB_q, 2H, J = 17.6 Hz, NHC$\underline{H}_2$).

HRMS (ES) 203.1404 (M^+-Cl). $C_9H_{19}N_2O_3$ requires 203.1396.

Methyl *N*-{*N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalanyl}glycinate (3.29a)

The pyrrole acid **3.16b** (51 mg, 0.37 mmol, 1 equiv) was coupled with the dipeptide **3.28a** (110 mg, 0.40 mmol, 1.1 equiv) by General Procedure M. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 4:1) to give **3.29a** (64 mg, 49%) as a cream solid.

mp decomp. 60-80°C, then melting at 209-211°C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 3.13 (dd, 1H, J = 9.5, 13.9 Hz, C$\underline{H}_{2a}$Ph), 3.44 (dd, 1H, J = 4.9, 14.2 Hz, C$\underline{H}_{2b}$Ph), 3.78 (s, 3H, $CO_2C\underline{H}_3$), 4.10 (d, 2H, J = 5.9 Hz, NHC$\underline{H}_2$), 5.06 (m, 1H, NHC$\underline{H}$), 7.05 (m, 1H, pyrrole $\underline{H}$3), 7.09 (m, 1H, pyrrole $\underline{H}$4), 7.29 (t, 1H, J = 7.3 Hz, Ar$\underline{H}$), 7.37 (t, 2H, J = 7.6 Hz, Ar$\underline{H}$), 7.45 (d, 2H, J = 7.3 Hz, Ar$\underline{H}$), 7.93 (s(br), 1H, N$\underline{H}$CH$_2$), 8.05 (d, 1H, J = 8.8 Hz, N$\underline{H}$CH), 9.80 (s, 1H, C$\underline{H}$O), 11.45 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR (acetone-d_6, 75 MHz) δ 37.8 (C$\underline{H}_2$Ph), 40.9 (NH$\underline{C}$H$_2$), 51.5 ($CO_2\underline{C}H_3$), 54.7 (NH$\underline{C}$H), 112.0 (pyrrole $\underline{C}$3), 118.4 (pyrrole $\underline{C}$4), 126.7, 128.5, 129.5, 138.1 (Ar$\underline{C}$), 132.1

(pyrrole C2), 134.7 (pyrrole C5), 159.8 (CONHCH), 170.2 (CO_2), 171.6 ($CONHCH_2$), 180.4 (CHO).

FTIR (KBr, diffuse refraction method) 1558.4, 1670.2, 1747.4, 2341.4, 2360.7, 3288.4.

HRMS (EI) 357.1329 (M^+). $C_{18}H_{19}N_3O_5$ requires 357.1325.

Methyl *N*-{*N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-leucyl}glycinate (3.29b)

The pyrrole acid **3.16b** (120 mg, 0.86 mmol, 1 equiv) was coupled with the dipeptide **3.28b** (227 mg, 0.95 mmol, 1.1 equiv) by General Procedure M. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 4:1), followed by further flash chromatography on silica (ethyl acetate/acetone, 2:1), to give **3.29b** (126 mg, 45%) as an orange solid.

mp decomp. >50°C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 0.93 (m, 6H, $CH(CH_3)_2$), 1.65-1.80 (m, 3H, CH_2CH), 3.65 (s, 3H, CO_2CH_3), 3.95 (d, 2H, J = 5.9 Hz, $NHCH_2$), 4.73 (m, 1H, NHCH), 7.00 (s, 2H, pyrrole H3 and H4), 7.76 (s(br), 1H, $NHCH_2$), 7.87 (d, 1H, J = 8.3 Hz, NHCH), 9.71 (s, 1H, CHO), 11.38 (s(br), 1H, pyrrole NH).

^{13}C NMR (acetone-d_6, 75 MHz) δ 21.2, 22.7 ($CH(CH_3)_2$), 24.8 (CH_2CH), 40.8 ($NHCH_2$), 40.9 (CH_2CH), 51.5 (CO_2CH_3), 51.9 (NHCH), 112.2 (pyrrole C3), 118.7 (pyrrole C4), 132.2 (pyrrole C2), 134.7 (pyrrole C5), 160.2 (CONHCH), 170.3 (CO_2CH_3), 172.8 ($CONHCH_2$), 180.5 (CHO).

FTIR (KBr, diffuse refraction method) 1554.5, 1670.2, 1747.4, 2958.6, 3255.6, 3413.8.

HRMS (ES) 346.1372 (M^++Na). $C_{15}H_{21}N_3NaO_5$ requires 346.1379.

Methyl *N*-{*N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalanyl}-L-leucinate (3.29c)

N-(L-Phenylalanyl)-L-leucine hydrate **3.27c** (200 mg, 0.67 mmol) was treated with diazomethane by General Procedure L to give methyl *N*-(L-phenylalanyl)-L-leucinate (**3.28c**, 202 mg) as a light brown oil. This dipeptide was coupled with the pyrrole acid **3.16b** (87 mg, 0.63 mmol, 1 equiv to 1.1 equiv of amine) by General Procedure M. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 3:1), followed by diffusion of petroleum ether into a solution of the title product dissolved in ethyl acetate, to give **3.29c** (135 mg, 52% over two steps) as a tan solid.

mp 100-103°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.85 (m, 6H, $CH(C\underline{H}_3)_2$), 1.43-1.61 (m, 3H, $C\underline{H}_2C\underline{H}$), 3.13 (m, 2H, $C\underline{H}_2Ph$), 3.73 (s, 3H, $CO_2C\underline{H}_3$), 4.56 (m, 1H, Leu α$\underline{H}$), 5.06 (q, 1H, J = 7.3 Hz, Phe α$\underline{H}$), 6.64 (dd, 1H, *J* = 2.4, 3.9 Hz, pyrrole $\underline{H}3$), 6.90 (m, 2H, pyrrole $\underline{H}4$ plus N$\underline{H}$CH), 7.19 (m, 6H, Ar$\underline{H}$ plus N$\underline{H}$CH), 9.61 (s, 1H, C$\underline{H}$O), 10.68 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 22.1, 22.6 ($CH(\underline{C}H_3)_2$), 24.8, 41.6 ($\underline{C}H_2C\underline{H}$), 38.8 ($\underline{C}H_2Ph$), 51.0 (Leu α$\underline{C}$H), 52.5 ($CO_2\underline{C}H_3$), 54.7 (Phe α$\underline{C}$H), 111.3 (pyrrole $\underline{C}3$), 119.6 (pyrrole $\underline{C}4$), 126.9, 128.4, 129.4, 136.4 (Ar$\underline{C}$), 131.3 (pyrrole $\underline{C}2$), 134.1 (pyrrole $\underline{C}5$), 159.7 (Pyr$\underline{C}$ONH), 171.3 (CH$\underline{C}$ONH), 173.1 ($\underline{C}O_2$), 180.3 ($\underline{C}$HO).

FTIR (KBr, diffuse refraction method) 1558.4, 1635.5, 1674.1, 1743.5, 2958.6, 3286.5.

HRMS (ES) 414.2031 (M^++H). $C_{22}H_{28}N_3O_5$ requires 414.2029.

Methyl *N*-{*N*-[(5-hydroxymethyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalanyl}-L-leucinate (3.25)

The pyrrole **3.29c** (82 mg, 0.20 mmol, 1 equiv) was reduced with lithium borohydride (9 mg, 0.40 mmol, 2 equiv) by General Procedure K. The crude product was purified by flash chromatography on silica (ethyl acetate), followed by diffusion of petroleum ether into a solution of the title product dissolved in ethyl acetate, to give **3.25** (56 mg, 68%) as a cream solid.

mp decomp. >85°C.

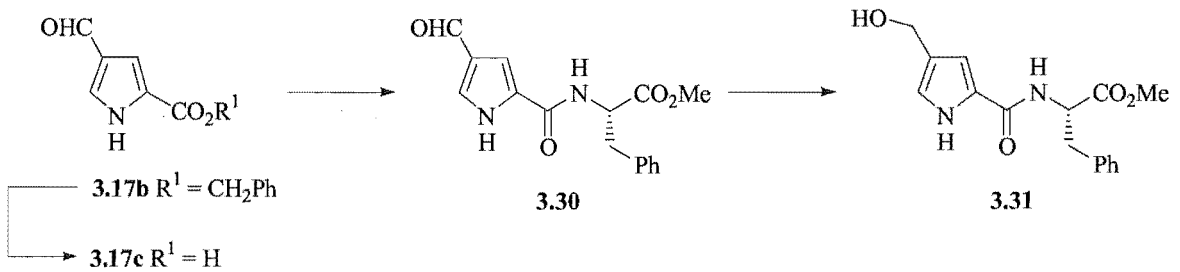
^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.84 (m, 6H, $CH(C\underline{H}_3)_2$), 1.44-1.59 (m, 3H, $C\underline{H}_2C\underline{H}$), 3.11 (m, 2H, $C\underline{H}_2Ph$), 3.69 (s, 3H, $CO_2C\underline{H}_3$), 4.59 (m, 3H, Leu α$\underline{H}$ plus $C\underline{H}_2OH$), 5.05 (q, 1H, *J* = 7.7 Hz, Phe α$\underline{H}$), 6.04 (t, 1H, *J* = 2.9 Hz, pyrrole $\underline{H}4$), 6.53 (m, 1H, pyrrole $\underline{H}3$), 7.03 (s(br), 1H, N$\underline{H}$CH), 7.16 (m, 5H, Ar$\underline{H}$), 7.25 (s(br), 1H, N$\underline{H}$CH), 10.41 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 22.0, 22.6 ($CH(\underline{C}H_3)_2$), 24.7, 41.5 ($\underline{C}H_2C\underline{H}$), 38.7 ($\underline{C}H_2Ph$), 50.8 (Leu α$\underline{C}$H), 52.3 ($CO_2\underline{C}H_3$), 54.5 (Phe α$\underline{C}$H), 57.5 ($\underline{C}H_2OH$), 108.1 (pyrrole $\underline{C}4$), 111.0 (pyrrole $\underline{C}3$), 124.9 (pyrrole $\underline{C}2$), 126.7, 128.4, 129.4, 136.3 (Ar$\underline{C}$), 136.7 (pyrrole $\underline{C}5$), 161.2 (Pyr$\underline{C}$ONH), 172.0 (CH$\underline{C}$ONH), 173.4 ($\underline{C}O_2$).

FTIR (KBr, diffuse refraction method) 1541.0, 1624.0, 1664.5, 1735.8, 2958.6, 3267.2.

HRMS (ES) 438.2023 (M^++Na). $C_{22}H_{29}N_3NaO_5$ requires 438.2005.

6.3.5 Attempted preparation of the 4-(hydroxymethyl)pyrrole 3.26



4-Formyl-1*H*-pyrrole-2-carboxylic acid (3.17c)

The pyrrole **3.17b**[26] (152 mg, 0.66 mmol, 1 equiv) was hydrolysed with potassium hydroxide (149 mg, 2.65 mmol, 4 equiv) by General Procedure F. Residual benzyl alcohol was removed by Kugelrohr distillation (up to 120°C) to give **3.17c** (71 mg, 77%) as a grey solid.

mp decomp. >215°C (lit.[24] 220°C).

^{1}H NMR (acetone-d_6, 300 MHz) δ 7.34 (m, 1H, pyrrole H3), 7.92 (m, 1H, pyrrole H5), 9.97 (s, 1H, CHO), 11.69 (s(br), 1H, pyrrole NH).

Methyl *N*-[(4-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate (3.30)

The pyrrole acid **3.17c** (58 mg, 0.42 mmol, 1 equiv) was coupled with L-phenylalanine methyl ester hydrochloride (99 mg, 0.46 mmol, 1.1 equiv) by modified General Procedure I. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 3:1) to give **3.30** (83 mg, 66%) as a tan solid.

mp 150-152°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.21 (m, 2H, CH_2Ph), 3.75 (s, 3H, CO_2CH_3), 5.02 (m, 1H, NHCH), 6.54 (d, 1H, J = 7.3 Hz, NHCH), 7.00 (dd, 1H, J = 1.5, 2.4 Hz, pyrrole H3), 7.12 (m, 2H, ArH), 7.27 (m, 3H, ArH), 7.51 (m, 1H, pyrrole H5), 9.80 (s, 1H, CHO), 10.41 (s(br), 1H, pyrrole NH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 37.9 ($\underline{C}H_2Ph$), 52.5 ($CO_2\underline{C}H_3$), 53.3 (NH$\underline{C}$H), 108.4 (pyrrole $\underline{C}$3), 127.3 (2xC, pyrrole $\underline{C}$2 and $\underline{C}$4), 128.7, 129.1, 135.5 (Ar$\underline{C}$), 129.0 (pyrrole $\underline{C}$5), 160.2 ($\underline{C}$ONH), 171.7 ($\underline{C}O_2$), 185.5 ($\underline{C}$HO).

FTIR (KBr, diffuse refraction method) 1535.2, 1629.7, 1654.8, 1735.8, 2362.6, 3211.3, 3334.7.

HRMS (ES) 301.1191 (M^++H). $C_{16}H_{17}N_2O_4$ requires 301.1188.

Methyl *N*-[(4-hydroxymethyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate (3.31)

The pyrrole **3.30** (43 mg, 0.14 mmol, 1 equiv) was reduced with lithium borohydride (6 mg, 0.29 mmol, 2 equiv) according to General Procedure K. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 10:1) to give **3.31** (12 mg, 28%) as a clear oil. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **3.31** dissolved in ethyl acetate.

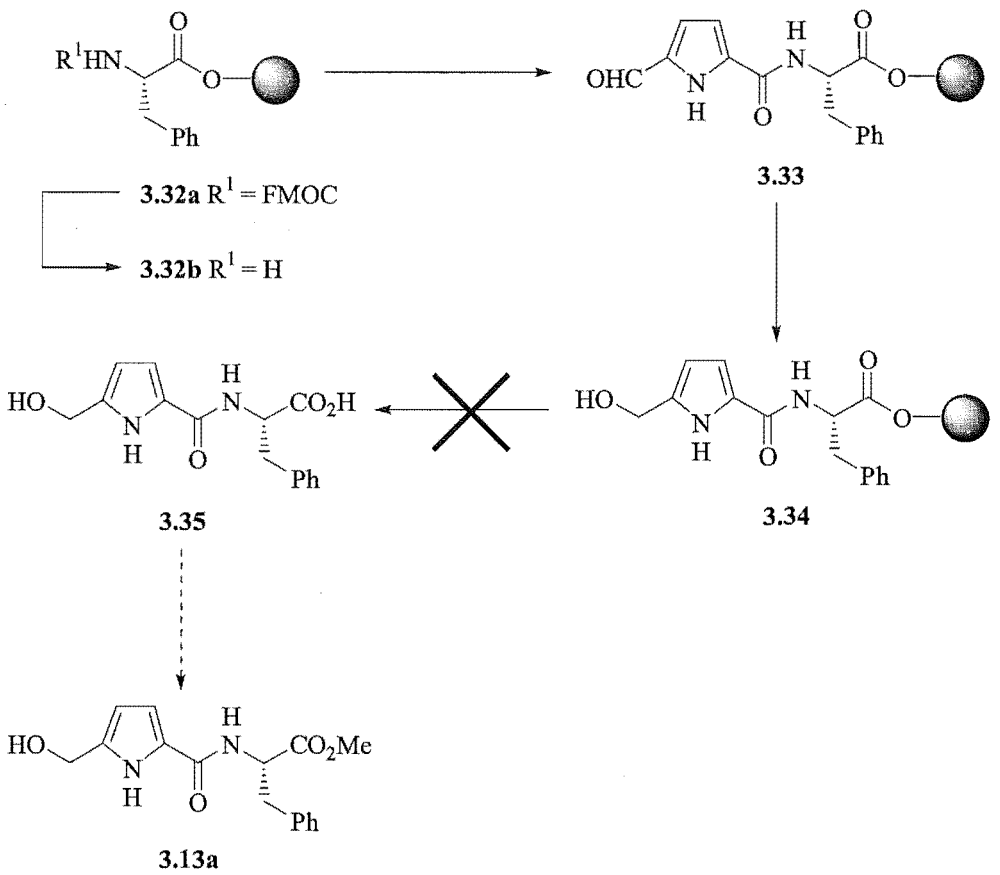
mp 112-114°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.19 (m, 2H, $C\underline{H}_2Ph$), 3.76 (s, 3H, $CO_2C\underline{H}_3$), 4.52 (s, 2H, $C\underline{H}_2OH$), 5.03 (m, 1H, NHC$\underline{H}$), 6.53 (m, 2H, N$\underline{H}$CH and pyrrole $\underline{H}$3), 6.84 (d, 1H, J = 1.5 Hz, pyrrole $\underline{H}$5), 7.15 (m, 2H, Ar$\underline{H}$), 7.27 (m, 3H, Ar$\underline{H}$), 9.41 (s(br), 1H, pyrrole N$\underline{H}$).

FTIR (KBr, diffuse refraction method) 1542.9, 1573.8, 1627.8, 1732.0, 1759.0, 2954.7, 3274.9, 3382.9.

No further analytical data was obtained due to product degradation.

6.3.6 Attempted preparation of the 5-(hydroxymethyl)pyrrole 3.13a by solid phase synthesis



***N*-[(5-Formyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalanine Wang resin (3.33)**

N-(9-Fluorenylmethoxycarbonyl)-L-phenylalanine Wang resin **3.32a** (GL Biochem (Shanghai) Ltd., 100-200 mesh, loading 0.42 mmol g^{-1}, 680 mg, 0.286 mmol, pre-dried under high vacuum for 16 h) was swollen with dry dichloromethane (5 mL) for 30 min, drained, and then re-suspended in 1:4 dry piperidine/dry dichloromethane (5 mL) and shaken at r.t. for 30 min. The resin was drained, washed with dry DMF (4 x 4 mL, 5 min), dry dichloromethane (3 x 4 mL, 5 min), and dried under high vacuum for 18 h. The resin was then swollen with dry dichloromethane (4 mL) for 30 min, drained, re-suspended in dry dichloromethane (4 mL), and the reaction syringe that contained the resin was shielded from light. Pyrrole acid **3.16b** (159 mg, 1.14 mmol, 4 equiv) and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (505 mg, 1.14 mmol, 4 equiv) were added, and the mixture was shaken at r.t. for 5 min. *N,N*-Diisopropylethylamine (369

mg, 2.86 mmol, 10 equiv) was then added, and shaking was continued for 48 h. The resin was drained, washed with dry DMF (4 x 4 mL, 5 min), dry ether (2 x 4 mL, 5 min), dry dichloromethane (3 x 4 mL, 5 min) and dried under high vacuum for 18 h.

_N_-[(5-Hydroxymethyl-1_H_-pyrrol-2-yl)carbonyl]-L-phenylalanine (3.35)

The resin **3.33** (loading 0.42 mmol g^{-1}, 260 mg, 0.109 mmol, pre-dried under high vacuum for 16 h) was swollen with dry dichloromethane (4 mL) for 1 h, drained, washed with dry DMF (4 mL, 5 min), and then re-suspended in dry DMF (4 mL). Sodium borohydride (41 mg, 1.09 mmol, 10 equiv) was added, and the resulting suspension was shaken for 24 h. The resin was drained, washed with dry DMF (3 x 4 mL, 5 min), dry ether (2 x 4 mL, 5 min), dry dichloromethane (3 x 4 mL, 5 min) and dried under high vacuum for 18 h. The resin was then swollen with dry dichloromethane (2 mL) for 30 min, drained, re-suspended in 1:1 dry TFA/dry dichloromethane (4 mL) and shaken at r.t. for 2 h. The resin was drained, washed with dry TFA (2 x 4 mL, 5 min), dry dichloromethane (2 x 4 mL, 5 min) and dry THF (2 x 4 mL, 5 min). The combined washings were dried by evaporation under reduced pressure. Analysis by ^{1}H NMR spectroscopy revealed that degradation had occurred.

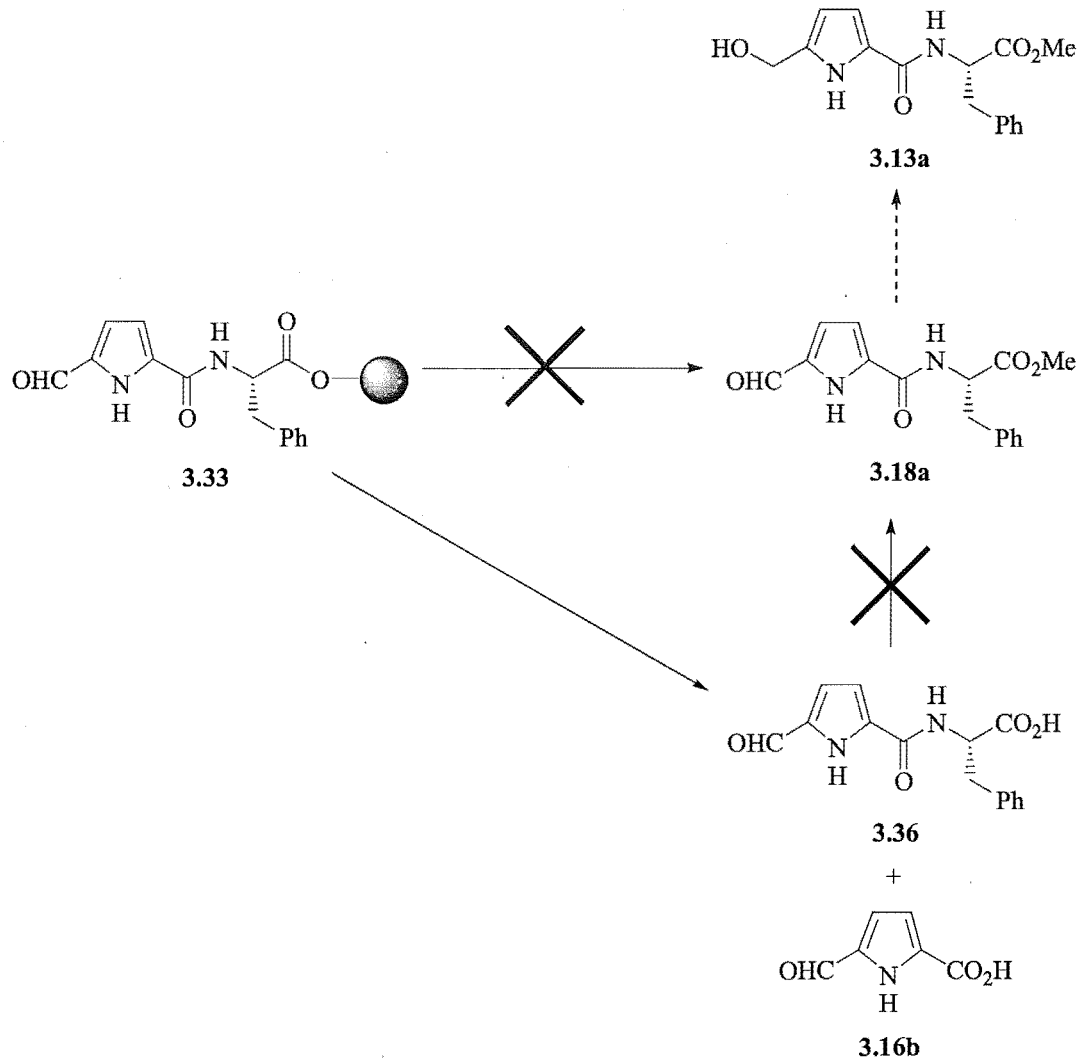
Determination of Stability of 3.13a in 1:1 Trifluoroacetic Acid/Dichloromethane

The pyrrole **3.13a** (2.0 mg) was dissolved in 1:1 dry TFA/dry dichloromethane (1 mL) and shaken at r.t. for 2 h. The solvent was removed by evaporation under reduced pressure to give a brown residue (4.9 mg). Analysis by ^{1}H NMR spectroscopy, thin layer chromatography and electrospray low resolution mass spectroscopy revealed degradation of pyrrole **3.13a**.

Determination of Stability of 3.18a in 1:1 Trifluoroacetic Acid/Dichloromethane

The pyrrole **3.18a** (25 mg) was dissolved in 1:1 dry TFA/dry dichloromethane (4 mL) and shaken at r.t. for 2 h. The solvent was removed by evaporation under reduced pressure to give a brown residue (29 mg). Analysis by ^{1}H NMR spectroscopy revealed no degradation of pyrrole **3.18a**.

6.3.7 Attempted preparation of the 5-formylpyrrole 3.18a by solid phase synthesis



Methyl *N*-[(5-formyl-1*H*-pyrrol-2-yl)carbonyl]-L-phenylalaninate (3.18a)

Method A

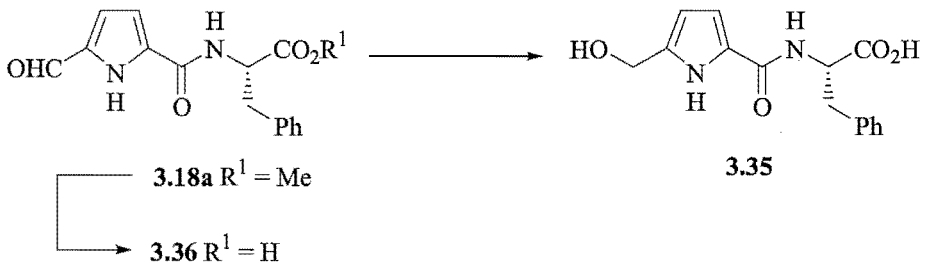
The resin **3.33** was suspended in a mixture of dry benzene (3 mL), dry methanol (0.5 mL) and dry triethylamine (0.1 mL). Potassium cyanide (approximately 2 mg) was added, and the resulting suspension was refluxed for 48 h. The beads were drained, then washed with dry dichloromethane (3 x 4 mL, 5 min), dry THF (2 x 4 mL, 5 min) and dry methanol (2 x 4 mL, 5 min). The combined filtrates were dried by evaporation under reduced pressure.

Analysis by ^{1}H NMR spectroscopy did not reveal the presence of product, in comparison to the ^{1}H NMR spectrum of **3.18a** previously prepared by solution phase chemistry.

Method B

The resin **3.33** was swollen with dry dichloromethane (4 mL) for 30 min, drained, re-suspended in 1:1 dry TFA/dry dichloromethane (4 mL) and shaken at r.t. for 2 h. The resin was drained, then washed with dry trifluoroacetic acid (2 x 4 mL, 5 min), dry THF (2 x 4 mL, 5 min) and dry dichloromethane (4 mL, 5 min). The combined filtrates were dried by evaporation under reduced pressure. Attempted purification by flash chromatography on silica (ethyl acetate/benzene, 1:4, then ethyl acetate/benzene, 1:2, visualised with Mary's dip) gave an approximately 1:1 mixture of the pyrrole acid **3.16b** and **3.36** (20 mg) by ^{1}H NMR spectroscopy. This mixture was treated with diazomethane by General Procedure L to give a complex mixture of materials by ^{1}H NMR spectroscopy, which could not be purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:3).

6.3.8 Attempted preparation of the hydroxymethylpyrrole 3.31 by solution phase synthesis



_N_-[(5-Formyl-1_H_-pyrrol-2-yl)carbonyl]-L-phenylalanine (3.36)

A stirred solution of the pyrrole **3.18a** (350 mg, 1.17 mmol, 1 equiv) and potassium carbonate (322 mg, 2.33 mmol, 2 equiv) in 10:1 methanol/water (27.5 mL) was refluxed for 18 h. After cooling to r.t., the methanol was removed by rotary evaporation, the resulting residue was dissolved in water (10 mL), 10% hydrochloric acid (10 mL) was added dropwise, and the resulting suspension was stirred for 15 min. Dichloromethane (20

mL) was added, stirring was continued for a further 15 min, and the layers were separated. The aqueous phase was extracted with dichloromethane (2 x 10 mL), the combined organic fractions were washed with saturated aqueous brine (10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. The crude product was purified by flash chromatography on silica (ethyl acetate/acetone, 5:1) to give **3.36** (274 mg, 82%) as a light orange solid.

mp decomp. >60°C.

^{1}H NMR (CD_3CN, 500 MHz) δ 3.14 (dd, 1H, J = 9.0, 13.9 Hz, C$\underline{H}_{2a}$Ph), 3.34 (dd, 1H, J = 5.4, 14.2 Hz, C$\underline{H}_{2b}$Ph), 4.87 (m, 1H, NHC$\underline{H}$), 6.83 (m, 1H, pyrrole $\underline{H3}$), 7.01 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole $\underline{H4}$), 7.31 (m, 6H, N$\underline{H}$CH and Ar$\underline{H}$), 9.67 (s, 1H, C$\underline{H}$O), 10.67 (s(br), 1H, pyrrole N$\underline{H}$).

^{13}C NMR (CD_3CN, 75 MHz) δ 37.7 ($\underline{C}H_2$Ph), 54.6 (NH$\underline{C}$H), 112.5 (pyrrole $\underline{C3}$), 119.9 (pyrrole $\underline{C4}$), 127.7, 129.4, 130.2, 138.2 (Ar$\underline{C}$), 132.2 (pyrrole $\underline{C2}$), 135.4 (pyrrole $\underline{C5}$), 160.8 ($\underline{C}$ONH), 173.1 ($\underline{C}O_2$H), 181.6 ($\underline{C}$HO).

FTIR (KBr, diffuse refraction method) 1560.3, 1654.8, 3051.2, 3433.1.

HRMS (EI) 286.0961 (M^+). $C_{16}H_{17}N_2O_4$ requires 286.0954.

_N_-[(5-Hydroxymethyl-1_H_-pyrrol-2-yl)carbonyl]-L-phenylalanine (3.35)

A stirred solution of the pyrrole **3.36** (82 mg, 0.29 mmol, 1 equiv) in 6:1 *iso*-propanol/water (3.5 mL) at 0°C was added sodium borohydride (33 mg, 0.85 mmol, 3 equiv) in two portions over 10 min. The resulting suspension was warmed to r.t. and stirred for 18 h, after which water (5 mL) was added, followed by acidification of the solution to pH ~1 by the dropwise addition of 1 M hydrochloric acid. The solution was extracted with ethyl acetate (3 x 10 mL), the combined organic fractions were washed with aqueous saturated brine (10 mL), dried ($MgSO_4$), and the solvent was removed by evaporation under reduced pressure. The crude product was purified by flash chromatography on silica (ethyl acetate/dichloromethane, 4:1, then ethyl acetate/acetone, 1:1, then methanol) to give **3.35** (36 mg, 44%) as a brown residue.

^{1}H NMR (500 MHz, CD_3OD) δ 3.07 (m, 1H, C$\underline{H}_{2a}$Ph), 3.26 (m, 1H, C$\underline{H}_{2b}$Ph), 4.44 (s, 2H, C$\underline{H}_2$OH), 4.80 (m, 1H, NHC$\underline{H}$), 6.09 (m, 1H, pyrrole $\underline{H4}$), 6.72 (d, 1H, J = 3.9 Hz, pyrrole $\underline{H3}$), 7.16-7.31 (m, 5H, Ar$\underline{H}$).

No further analytical data was obtained due to product degradation.

6.3.9 α-Chymotrypsin assay and results

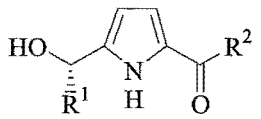
General Assay Conditions

See Section 6.2.5 earlier in this chapter for a summary of the General Assay Conditions.

α-Chymotrypsin Assay

Solutions of the pyrroles **3.13a-b**, **3.14a**, **3.24** and **3.25** were made to 1250 μg mL^{-1}, 125 μg mL^{-1} and 12.5 μg mL^{-1} in methanol. Tris-HCl (50 μL of a 0.4 M solution in water, pH 7.6), distilled water (50 μL), inhibitor solution (50 μL) and α-chymotrypsin (50 μL, Sigma ex-Bovine pancreas, 9 units mL^{-1} in 50 mM Tris-HCl, pH 7.6) were added to each well of the microtitre plate. Incubation at 37°C for 30 min was followed by the addition of *N*-succinyl-L-phenylalanine-4-nitroanilide (100 μL, 1 mg mL^{-1} solution in 50 mM Tris-HCl buffer, pH 7.6). The absorbance was read at 405 nm at hourly intervals until the maximum measured absorbance exceeded 1.00 absorbance units. Each inhibitor concentration was assayed in triplicate and average absorbances were used to calculate the percentage (%) inhibition. The calculated percent inhibition values are a measure of the enzyme activity relative to that when no enzyme was present (no substrate turnover, 100% "inhibition") and when no inhibitor was present (full substrate turnover, 0% inhibition). To this end, sample blanks to determine the absorbance for each inhibitor concentration in the absence of enzyme (*i.e.* no substrate turnover) were run concurrently, in which 50 mM Tris-HCl buffer, pH 7.6, replaced α-chymotrypsin. Maximum substrate turnover was determined by averaging the absorbance readings from six wells in which methanol replaced the inhibitor. All percentage (%) inhibition figures reported are rounded to the nearest 5%. The IC_{50} for **3.25** was determined was performing the assay in the same general manner as for the initial α-chymotrypsin assay, except that solutions of **3.25** were made to 12.0 mM, 3.01 mM, 0.75 mM, 0.19 mM, 47.0 μM, 11.8 μM, 2.94 μM and 0.73 μM. Each inhibitor

concentration was assayed in triplicate. The IC_{50} value was calculated by fitting the inhibitor concentration-percent inhibition data into Microsoft Excel®.



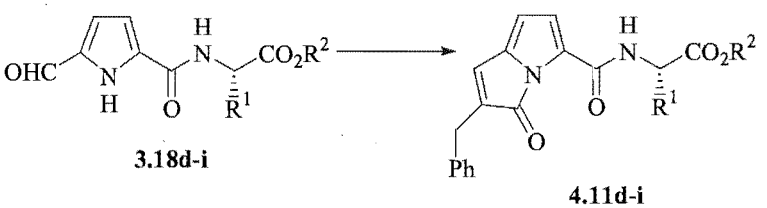
No.	R^1	R^2	% Inhibition ($\mu g\ mL^{-1}$)		
			1250	125	12.5
3.13a	H	NH-L-PheOMe	75	35	45
3.13b	H	NH-L-LeuOMe	10	5	20
3.14a	$Ph(CH_2)_2$	NH-L-PheOMe	75	35	15
3.24	H	N-L-ProOMe	45	25	5
3.25[¶]	H	NH-L-Phe-L-LeuOMe	100	20	15

[¶] $IC_{50} = 108\ \mu M$.

Table 6.2. Inhibition of α-chymotrypsin by **3.13a-b**, **3.14a**, **3.24** and **3.25**.

6.4 EXPERIMENTAL WORK DESCRIBED IN CHAPTER FOUR

6.4.1 Preparation of the pyrrolizinones 4.11d-i



	R^1	R^2
3.18d/4.11d	H	Bn
3.18e/4.11e	CH_2Ph	Et
3.18f/4.11f	CH_2CHMe_2	Et
3.18g/4.11g	$CHMe_2$	Me
3.18h/4.11h	Me	Me
3.18i/4.11i	H	Me

Benzyl *N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]glycinate (4.11d)

The pyrrole **3.18d** (60 mg, 0.21 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (71 mg, 0.42 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:3), followed by removal of residual hydrocinnamic acid by Kugelrohr distillation (up to 160-170°C), to give **4.11d** (35 mg, 42%) as an orange solid. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **4.11d** dissolved in ethyl acetate.

mp 138-139°C.

UV (CH_3CN) λ_{max} 299 nm ($\varepsilon = 5600\ M^{-1}cm^{-1}$), 431 nm ($\varepsilon = 1530\ M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 3.61 (d, 2H, J = 1.5 Hz, $C\underline{H}_2Ph$), 4.25 (d, 2H, J = 4.9 Hz, $NHC\underline{H}_2$), 5.21 (s, 2H, $CO_2C\underline{H}_2Ph$), 5.97 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H7), 6.63 (t, 1H, J = 1.7 Hz, pyrrolizin-3-one H1), 6.88 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H6), 7.25 (m, 4H, ArH), 7.34 (m, 6H, ArH), 9.34 (s(br), 1H, $N\underline{H}CH_2$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 31.9 ($\underline{C}H_2Ph$), 41.7 ($NH\underline{C}H_2$), 67.1 ($CO_2\underline{C}H_2Ph$), 111.1 (pyrrolizin-3-one C7), 124.4 (pyrrolizin-3-one C6), 126.9, 128.0, 128.4, 128.6, 128.8, 128.8, 135.3, 136.9 (Ar$\underline{C}$), 130.3 (pyrrolizin-3-one C5), 132.3 (pyrrolizin-3-one C1), 137.0 (pyrrolizin-3-one C2), 139.8 (pyrrolizin-3-one C8), 158.3 ($\underline{C}ONH$), 167.8 (pyrrolizin-3-one C3), 169.5 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1562.2, 1581.5, 1647.1, 1726.2, 3033.8, 3284.5.

HRMS (EI) 400.1431 (M^+). $C_{24}H_{20}N_2O_4$ requires 400.1423.

Micro. Calcd for $C_{24}H_{20}N_2O_4$: C, 71.99; H, 5.03; N, 7.00. Found: C, 71.82; H, 5.20; N, 7.24.

Ethyl *N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]-L-phenylalaninate (4.11e)

The pyrrole **3.18e** (40 mg, 0.13 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (43 mg, 0.25 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ether/petroleum ether, 3:1), followed by removal of residual hydrocinnamic acid by Kugelrohr distillation (up to 160-200°C), to give **4.11e** (19 mg, 35%) as a red oil.

UV (CH_3CN) λ_{max} 299 nm (ε = 8500 $M^{-1}cm^{-1}$), 432 nm (ε = 1920 $M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 1.20 (t, 3H, J = 7.3 Hz, $CO_2CH_2C\underline{H}_3$), 3.14 (m, 2H, $CHC\underline{H}_2Ph$), 3.59 (s, 2H, $C\underline{H}_2Ph$), 4.16 (q, 2H, J = 7.0 Hz, $CO_2C\underline{H}_2CH_3$), 4.94 (m, 1H, NHC<u>H</u>), 5.93 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one <u>H7</u>), 6.60 (t, 1H, J = 1.5 Hz, pyrrolizin-3-one <u>H1</u>), 6.83 (d, 1H, J = 2.9 Hz, pyrrolizin-3-one <u>H6</u>), 7.24 (m, 8H, Ar<u>H</u>), 7.34 (m, 2H, Ar<u>H</u>), 9.32 (d, 1H, J = 7.3 Hz, N<u>H</u>CH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1 ($CO_2CH_2\underline{C}H_3$), 31.9 ($\underline{C}H_2Ph$), 38.2 ($CH\underline{C}H_2Ph$), 54.3 (NH<u>C</u>H), 61.3 ($CO_2\underline{C}H_2CH_3$), 110.8 (pyrrolizin-3-one <u>C7</u>), 124.3 (pyrrolizin-3-one <u>C6</u>), 126.8, 126.9, 128.4, 128.8, 128.9, 129.3, 136.3, 136.9 (Ar<u>C</u>), 130.4 (pyrrolizin-3-one <u>C5</u>), 132.2 (pyrrolizin-3-one <u>C1</u>), 137.1 (pyrrolizin-3-one <u>C2</u>), 139.7 (pyrrolizin-3-one <u>C8</u>), 157.7 (<u>C</u>ONH), 167.6 (pyrrolizin-3-one <u>C3</u>), 171.5 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1556.4, 1579.6, 1643.2, 1728.1, 3012.6, 3030.0, 3271.0.

HRMS (EI) 428.1750 (M^+). $C_{26}H_{24}N_2O_4$ requires 428.1736.

Ethyl *N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]-L-leucinate (4.11f)

The pyrrole **3.18f** (60 mg, 0.21 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (72 mg, 0.43 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:3), followed by removal of residual hydrocinnamic acid and starting material by Kugelrohr distillation (up to 160-220°C), to give **4.11f** (26 mg, 31%) as an orange oil. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **4.11f** dissolved in ethyl acetate.

mp 104-105°C.

UV (CH_3CN) λ_{max} 298 nm ($\varepsilon = 7450\ M^{-1}cm^{-1}$), 429 nm ($\varepsilon = 1580\ M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 0.96 (m, 6H, $CH(C\underline{H}_3)_2$), 1.27 (t, 3H, J = 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.73 (m, 3H, $C\underline{H}_2C\underline{H}$), 3.60 (s, 2H, $C\underline{H}_2Ph$), 4.19 (q, 2H, J = 7.2 Hz, $CO_2C\underline{H}_2CH_3$), 4.69 (m, 1H, NHC<u>H</u>), 5.94 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one <u>H7</u>), 6.59 (t, 1H, J = 1.7 Hz, pyrrolizin-3-one <u>H1</u>), 6.85 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one <u>H6</u>), 7.25 (m, 3H, Ar<u>H</u>), 7.33 (m, 2H, Ar<u>H</u>), 9.21 (d, 1H, J = 7.3 Hz, N<u>H</u>CH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2 ($CO_2CH_2\underline{C}H_3$), 21.9, 22.9 ($CH(\underline{C}H_3)_2$), 25.0, 41.2 ($\underline{C}H_2CH$), 31.9 ($\underline{C}H_2Ph$), 51.4 (NH<u>C</u>H), 61.2 ($CO_2\underline{C}H_2CH_3$), 111.0 (pyrrolizin-3-one <u>C7</u>), 124.3 (pyrrolizin-3-one <u>C6</u>), 126.9, 128.8, 128.9, 136.9 (Ar<u>C</u>), 130.6 (pyrrolizin-3-one <u>C5</u>), 132.3 (pyrrolizin-3-one <u>C1</u>), 137.0 (pyrrolizin-3-one <u>C2</u>), 139.7 (pyrrolizin-3-one <u>C8</u>), 157.9 (<u>C</u>ONH), 167.9 (pyrrolizin-3-one <u>C3</u>), 172.7 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1560.3, 1581.5, 1643.2, 1728.1, 3037.7, 3274.9.

HRMS (EI) 394.1887 (M^+). $C_{23}H_{26}N_2O_4$ requires 394.1893.

Micro. Calcd for $C_{23}H_{26}N_2O_4$: C, 70.03; H, 6.64; N, 7.10. Found: 70.20; H, 6.68; N, 7.39.

Methyl *N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]-L-valinate (4.11g)

The pyrrole **3.18g** (25 mg, 0.10 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (33 mg, 0.20 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ether/petroleum ether, 3:1), followed by removal of residual hydrocinnamic acid by Kugelrohr distillation (up to 160-200°C), to give **4.11g** (16 mg, 44%) as a red oil. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **4.11g** dissolved in ethyl acetate.

mp 93-94°C.

UV (CH_3CN) λ_{max} 300 nm ($\varepsilon = 7580\ M^{-1}cm^{-1}$), 434 nm ($\varepsilon = 1780\ M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 1.03 (m, 6H, $CH(C\underline{H}_3)_2$), 2.31 (m, 1H, $C\underline{H}(CH_3)_2$), 3.61 (d, 2H, J = 1.5 Hz, $C\underline{H}_2Ph$), 3.74 (s, 3H, $CO_2C\underline{H}_3$), 4.66 (m, 1H, NHC<u>H</u>), 5.94 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one <u>H7</u>), 6.58 (t, 1H, J = 1.7 Hz, pyrrolizin-3-one <u>H1</u>), 6.86 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one <u>H6</u>), 7.25 (m, 3H, Ar<u>H</u>), 7.33 (m, 2H, Ar<u>H</u>), 9.26 (d, 1H, J = 7.8 Hz, N<u>H</u>CH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.8, 19.2 ($CH(\underline{C}H_3)_2$), 30.8 ($\underline{C}H(CH_3)_2$), 31.9 ($\underline{C}H_2Ph$), 52.1 ($CO_2\underline{C}H_3$), 57.9 (NH<u>C</u>H), 111.0 (pyrrolizin-3-one <u>C7</u>), 124.4 (pyrrolizin-3-one <u>C6</u>),

126.9, 128.8, 128.9, 137.0 (ArC), 130.6 (pyrrolizin-3-one C5), 132.3 (pyrrolizin-3-one C1), 137.0 (pyrrolizin-3-one C2), 139.8 (pyrrolizin-3-one C8), 158.1 (CONH), 167.9 (pyrrolizin-3-one C3), 172.2 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1560.3, 1581.5, 1643.2, 1728.1, 3020.3, 3030.0.

HRMS (EI) 366.1581 (M^+). $C_{21}H_{22}N_2O_4$ requires 366.1580.

Micro. Calcd for $C_{21}H_{22}N_2O_4$: C, 68.84; H, 6.05; N, 7.65. Found: C, 68.71; H, 6.00; N, 7.55.

Methyl *N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]-L-alaninate (4.11h)

The pyrrole **3.18h** (77 mg, 0.34 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (116 mg, 0.69 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ether/petroleum ether, 5:1), followed by removal of residual hydrocinnamic acid by Kugelrohr distillation (up to 160-200°C), then flash chromatography on silica (ethyl acetate/petroleum ether, 1:1), to give **4.11h** (24 mg, 21%) as an orange oil. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **4.11h** dissolved in ethyl acetate. Crystals suitable for X-ray crystallography were obtained. See Appendix for data.

mp 93-95°C.

UV (CH_3CN) λ_{max} 299 nm ($\varepsilon = 8950\ M^{-1}cm^{-1}$), 434 nm ($\varepsilon = 2190\ M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 1.52 (d, 3H, J = 7.3 Hz, $CHC\underline{H}_3$), 3.61 (s, 2H, $C\underline{H}_2Ph$), 3.76 (s, 3H, $CO_2C\underline{H}_3$), 4.71 (m, 1H, NHC<u>H</u>), 5.95 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H7), 6.61 (s, 1H, pyrrolizin-3-one H1), 6.86 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H6), 7.25 (m, 3H, Ar<u>H</u>), 7.33 (m, 2H, Ar<u>H</u>), 9.31 (d, 1H, J = 6.3 Hz, N<u>H</u>CH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 18.0 ($CH\underline{C}H_3$), 31.9 ($\underline{C}H_2Ph$), 48.5 (NH<u>C</u>H), 52.4 ($CO_2\underline{C}H_3$), 111.0 (pyrrolizin-3-one C7), 124.3 (pyrrolizin-3-one C6), 126.9, 128.8, 128.9, 136.9 (ArC), 130.5 (pyrrolizin-3-one C5), 132.3 (pyrrolizin-3-one C1), 137.0 (pyrrolizin-3-one C2), 139.7 (pyrrolizin-3-one C8), 157.6 (CONH), 167.9 (pyrrolizin-3-one C3), 173.2 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1581.5, 1647.1, 1726.2, 3026.3.

HRMS (EI) 338.1274 (M^+). $C_{19}H_{18}N_2O_4$ requires 338.1267.

Micro. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.45; H, 5.36; N, 8.28. Found: C, 67.42; H, 5.58; N, 7.99.

Methyl *N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]glycinate (4.11i)

The pyrrole **3.18i** (35 mg, 0.17 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (56 mg, 0.33 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 2:1) to give **4.11i** (23 mg, 43%) as an orange solid. An analytical sample was obtained by recrystallisation from ethyl acetate/petroleum ether to give orange plates.

mp 149-150°C.

UV (CH_3CN) λ_{max} 297 nm ($\varepsilon = 7190\ M^{-1}cm^{-1}$), 433 nm ($\varepsilon = 1860\ M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 3.61 (s, 2H, $C\underline{H}_2Ph$), 3.77 (s, 3H, $CO_2C\underline{H}_3$), 4.20 (d, 2H, J = 5.4 Hz, $NHC\underline{H}_2$), 5.96 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H7), 6.61 (m, 1H, pyrrolizin-3-one H1), 6.87 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H6), 7.25 (m, 3H, Ar$\underline{H}$), 7.33 (m, 2H, Ar$\underline{H}$), 9.32 (s(br), 1H, $N\underline{H}CH_2$).

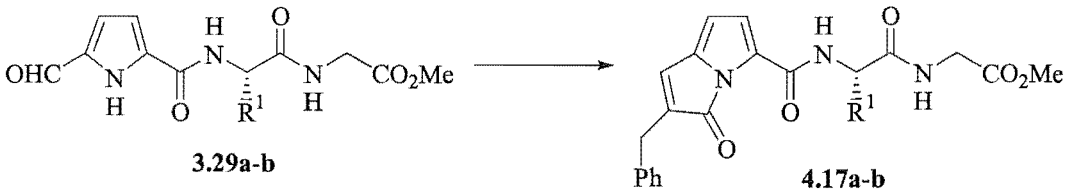
^{13}C NMR ($CDCl_3$, 75 MHz) δ 32.0 ($\underline{C}H_2Ph$), 41.5 ($NH\underline{C}H_2$), 52.3 ($CO_2\underline{C}H_3$), 111.1 (pyrrolizin-3-one C7), 124.4 (pyrrolizin-3-one C6), 126.9, 128.8, 128.8, 136.9 (Ar$\underline{C}$), 130.3 (pyrrolizin-3-one C5), 132.3 (pyrrolizin-3-one C1), 137.0 (pyrrolizin-3-one C2), 139.8 (pyrrolizin-3-one C8), 158.3 ($\underline{C}ONH$), 167.9 (pyrrolizin-3-one C3), 170.1 ($\underline{C}O_2$).

FTIR ($CHCl_3$) 1562.2, 1581.5, 1647.1, 1724.2, 3014.5, 3294.4.

HRMS (EI) 324.1110 (M^+). $C_{18}H_{16}N_2O_4$ requires 324.1110.

Micro. Calcd for $C_{18}H_{16}N_2O_4$: C, 66.66; H, 4.97; N, 8.64. Found: C, 66.73; H, 5.22; N, 8.65.

6.4.2 Preparation of the pyrrolizinones 4.17a-b



3.29a-b **4.17a-b**

	R^1
3.29a/4.17a	CH_2Ph
3.29b/4.17b	CH_2CHMe_2

Methyl *N*-{*N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]-L-phenylalanyl}glycinate (4.17a)

The pyrrole **3.29a** (31 mg, 0.09 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (29 mg, 0.17 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 7:1), followed by further flash chromatography on silica (ethyl acetate/dichloromethane, 3:1), to give **4.17a** (14 mg, 34%) as a red oil. An analytical sample was obtained by trituration with 1:1 ethyl acetate/petroleum ether to give a red solid.

mp 149-152°C.

UV (CH_3CN) λ_{max} 299 nm ($\varepsilon = 5500\ M^{-1}cm^{-1}$), 432 nm ($\varepsilon = 1570\ M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 3.11 (dd, 1H, J = 8.5, 13.9 Hz, $CHCH_{2a}Ph$), 3.30 (dd, 1H, J = 6.3, 14.2 Hz, $CHCH_{2b}Ph$), 3.60 (s, 2H, CH_2Ph), 3.70 (s, 3H, CO_2CH_3), 3.99 (m, 2H, $NHCH_2$), 4.80 (q, 1H, J = 7.2 Hz, NHCH), 5.93 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H7), 6.61 (s, 1H, pyrrolizin-3-one H1), 6.68 (s(br), 1H, $NHCH_2$), 6.82 (d, 1H, J = 3.4 Hz, pyrrolizin-3-one H6), 7.18-7.36 (m, 10H, ArH), 9.34 (d, 1H, J = 7.3 Hz, NHCH).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 31.9 (CH_2Ph), 37.4 ($CHCH_2Ph$), 41.2 ($NHCH_2$), 52.3 (CO_2CH_3), 55.4 (NHCH), 111.0 (pyrrolizin-3-one C7), 124.6 (pyrrolizin-3-one C6), 126.8, 126.9, 128.5, 128.8, 128.9, 129.3, 136.9, 136.9 (ArC), 130.0 (pyrrolizin-3-one C5), 132.2 (pyrrolizin-3-one C1), 137.0 (pyrrolizin-3-one C2), 139.9 (pyrrolizin-3-one C8), 158.4 (CONHCH), 167.6 (pyrrolizin-3-one C3), 170.1 (CO_2), 171.1 ($CONHCH_2$).

FTIR (KBr, diffuse refraction method) 1523.7, 1583.4, 1643.2, 1668.3, 1716.5, 1749.3, 2923.9, 3026.1, 3085.9, 3261.4, 3315.4.

HRMS (EI) 471.1792 (M^+). $C_{27}H_{25}N_3O_5$ requires 471.1795.

Methyl *N*-{*N*-[(2-benzyl-3-oxo-3*H*-pyrrolizin-5-yl)carbonyl]-L-leucyl}glycinate (4.17b)

The pyrrole **3.29b** (50 mg, 0.15 mmol, 1 equiv) was treated with hydrocinnamoyl chloride (52 mg, 0.31 mmol, 2 equiv) by General Procedure N. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 7:1), followed by further flash chromatography on silica (ethyl acetate/dichloromethane, 4:1), to give **4.17b** (22 mg, 33%) as a red oil. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **4.17b** dissolved in ethyl acetate.

mp 134-137°C.

UV (CH_3CN) λ_{max} 298 nm ($\varepsilon = 7100\ M^{-1}cm^{-1}$), 435 nm ($\varepsilon = 1840\ M^{-1}cm^{-1}$).

^1H NMR ($CDCl_3$, 500 MHz) δ 0.95 (m, 6H, $CH(CH_3)_2$), 1.73 (m, 2H, $CH_{2a}CH$), 1.85 (m, 1H, $CH_{2b}CH$), 3.60 (s, 2H, CH_2Ph), 3.72 (s, 3H, CO_2CH_3), 4.03 (m, 2H, $NHCH_2$), 4.57 (q, 1H, $J = 7.2$ Hz, NHCH), 5.96 (d, 1H, $J = 3.4$ Hz, pyrrolizin-3-one H7), 6.60 (d, 1H, $J = 1.5$ Hz, pyrrolizin-3-one H1), 6.86 (d, 2H, $J = 2.9$ Hz, pyrrolizin-3-one H6, $NHCH_2$), 7.24 (m, 3H, ArH), 7.33 (m, 2H, ArH), 9.17 (d, 1H, $J = 6.8$ Hz, NHCH).

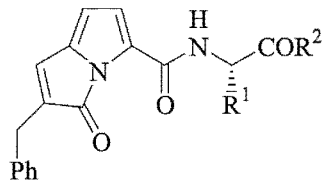
^{13}C NMR ($CDCl_3$, 75 MHz) δ 21.9, 23.0 ($CH(CH_3)_2$), 24.8 (CH_2CH), 31.9 (CH_2Ph), 39.9 (CH_2CH), 41.2 ($NHCH_2$), 52.1 (NHCH), 52.3 (CO_2CH_3), 111.1 (pyrrolizin-3-one C7), 124.6 (pyrrolizin-3-one C6), 126.9, 128.8, 128.9, 136.9 (ArC), 130.2 (pyrrolizin-3-one C5), 132.3 (pyrrolizin-3-one C1), 137.0 (pyrrolizin-3-one C2), 139.9 (pyrrolizin-3-one C8), 158.5 (CONHCH), 167.9 (pyrrolizin-3-one C3), 170.2 (CO_2), 172.0 ($CONHCH_2$).

FTIR (KBr, diffuse refraction method) 1527.5, 1583.4, 1641.3, 1666.4, 1720.4, 1743.5, 1751.2, 2954.7, 3327.0.

HRMS (EI) 437.1951 (M^+). $C_{24}H_{27}N_3O_5$ requires 437.1951.

6.4.3 UV spectroscopic analysis of the pyrrolizinones 4.11a-i and 4.17a-b

Ultraviolet spectra for the pyrrolizinones **4.11a-i** and **4.17a-b** were obtained using a GBC 920 UV/VIS spectrophotometer interfaced with an Intel 486 PC operating GBC's Spectral software version 1.5. Spectra were obtained in CH_3CN, and ε values were calculated from absorbances below 1.00 absorbance unit by dilution of the stock solutions of **4.11a-i** and **4.17a-b** in CH_3CN where necessary.



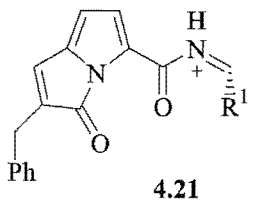
No.	R^1	R^2	λ_{max} (nm) [ε (M^{-1} cm^{-1})]	Ratio $\varepsilon_{(\sim 300\ nm)}/\varepsilon_{(\sim 430\ nm)}$
4.11a[#]	CH_2Ph	OMe	299 [6380], 434 [1950]	3.27
4.11b[#]	CH_2CHMe_2	OMe	300 [5010], 434 [1550]	3.23
4.11c[#]	H	OEt	299 [8200], 433 [2150]	3.81
4.11d	H	OBn	299 [5610], 431 [1530]	3.67
4.11e	CH_2Ph	OEt	299 [8500], 432 [1920]	4.45
(3.18e)	CH_2Ph	OEt	291 [28610]	na*
4.11f	CH_2CHMe_2	OEt	298 [7450], 429 [1580]	4.71
4.11g	$CHMe_2$	OMe	300 [7580], 434 [1780]	4.25
4.11h	Me	OMe	299 [8950], 434 [2190]	4.09
4.11i	H	OMe	297 [7190], 433 [1860]	3.86
(3.18i)	H	OMe	289 [16780]	na*
4.17a	CH_2Ph	$NHCH_2CO_2Me$	299 [5500], 432 [1570]	3.50
4.17b	CH_2CHMe_2	$NHCH_2CO_2Me$	298 [7100], 435 [1840]	3.86

[#] Previously synthesised[27]
* Not applicable

Table 6.3. UV spectral data for pyrrolizinones **4.11a-i** and **4.17a-b** in acetonitrile. Data for **3.18e** and **3.18i** is included underneath their respective analogues **4.11e** and **4.11i**.

6.4.4 Fragmentation analysis of the pyrrolizinones 4.11a-i and 4.17a-b by LRMS

LRMS analysis of the pyrrolizinones **4.11a-i** and **4.17a-b** was performed by electron impact ionisation mass spectrometry (see Section 6.1 for details).



4.21

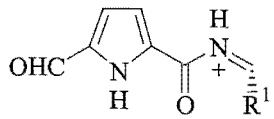
No.	R^1	*m/z* of $M^{+\cdot}$	*m/z* of ion **4.21** (% intensity)	$M^{+\cdot}$ - (*m/z* **4.21**)	$M^{+\cdot}$ - (*m/z* **4.21**) corresponds to loss of:
4.11a[#]	CH_2Ph	414.2	355.1 (6)	59.1	$-CO_2Me$
4.11b[#]	CH_2CHMe_2	380.2	321.2 (31)	59.0	$-CO_2Me$
4.11c[#]	H	338.1	265.1 (7)	73.0	$-CO_2Et$
4.11d	H	400.1	265.1 (8)	135.0	$-CO_2CH_2Ph$
4.11e	CH_2Ph	428.2	355.1 (7)	73.1	$-CO_2Et$
4.11f	CH_2CHMe_2	394.2	321.2 (76)	73.0	$-CO_2Et$
4.11g	$CHMe_2$	366.2	307.1 (46)	59.1	$-CO_2Me$
4.11h	Me	338.1	279.1 (40)	59.0	$-CO_2Me$
4.11i	H	324.1	265.1 (8)	59.0	$-CO_2Me$
4.17a	CH_2Ph	471.2	355.2 (42)	116.0	$-CONHCH_2CO_2Me$
4.17b	CH_2CHMe_2	437.2	321.2 (81)	116.0	$-CONHCH_2CO_2Me$

[#] = Previously synthesised[27]

Table 6.4. LRMS data for **4.11a-i** and **4.17a-b** to give product ion **4.21**.

6.4.4 Fragmentation analysis of the 5-formylpyrroles 3.18a-i and 3.29a-b by LRMS

LRMS analysis of the 5-formylpyrroles **3.18a-i** and **3.29a** was performed by electron impact ionisation mass spectrometry, with the exception of **3.29b**, which was analysed by electrospray ionisation mass spectrometry (see Section 6.1 for details).



4.27

No.	R^1	*m/z* of $M^{+\cdot}$	*m/z* of ion **4.27** (% intensity)	$M^{+\cdot}$ - (*m/z* **4.27**)	$M^{+\cdot}$ - (*m/z* **4.27**) corresponds to loss of:
3.18a*	CH_2Ph	300.1	241.1 (5)	59.0	$-CO_2Me$
3.18b	CH_2CHMe_2	266.1	207.1 (38)	59.0	$-CO_2Me$
3.18c#	H			73.0	$-CO_2Et$
3.18d	H	286.1	151.1 (18)	135.0	$-CO_2CH_2Ph$
3.18e	CH_2Ph	314.1	241.1 (6)	73.0	$-CO_2Et$
3.18f	CH_2CHMe_2	280.1	207.1 (47)	73.0	$-CO_2Et$
3.18g	$CHMe_2$	252.1	193.1 (44)	59.1	$-CO_2Me$
3.18h	Me	224.1	165.0 (98)	59.1	$-CO_2Me$
3.18i	H	210.1	151.1 (25)	59.0	$-CO_2Me$
3.29a	CH_2Ph	357.1	241.1 (25)	116.0	$-CONHCH_2CO_2Me$
3.29b	CH_2CHMe_2	346.1	207.1 (12)	139.0	$-CONHCH_2CO_2Me - Na^+$

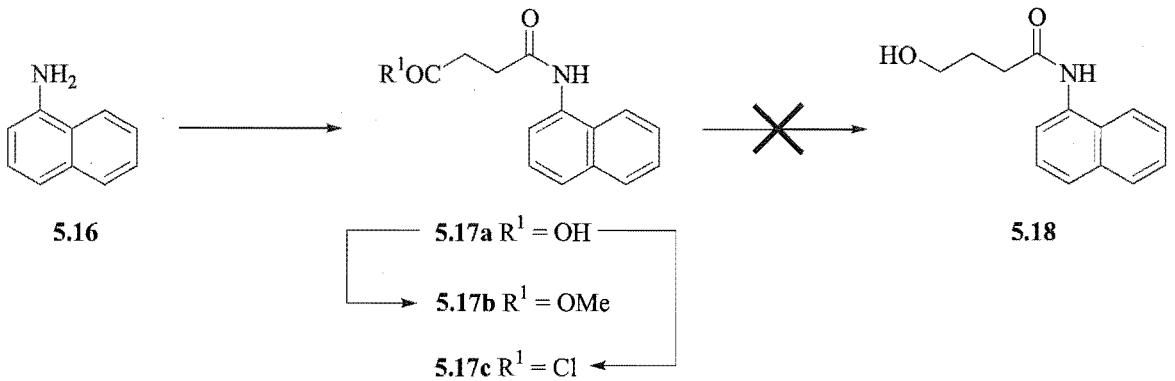
* = Synthesised by author, but mass spectrometry results were obtained from the previous study[27]

= Previously synthesised[27]

Table 6.5. LRMS data for **3.18a-i** and **3.29a-b** to give product ion **4.27**.

EXPERIMENTAL WORK DESCRIBED IN CHAPTER FIVE

6.5.1 Attempted preparation of the alcohol 5.18 by reduction of the acid 5.17a and derivatives



***N*-(Naphthalen-1-yl)succinamic acid (5.17a)**

Naphthalen-1-ylamine **5.16** (1.43 g, 10 mmol, 1 equiv) was treated with succinic anhydride (1.0 g, 10 mmol, 1 equiv) by General Procedure P. After cooling to r.t., the solid was collected by filtration and washed with cold petroleum ether. The material was dissolved in ethyl acetate (60 mL) and 5% aqueous sodium hydroxide (60 mL), the layers were separated, and the organic phase was extracted with 5% aqueous sodium hydroxide (20 mL). The combined aqueous fractions were acidified to pH ~1 with concentrated aqueous hydrochloric acid, and the solid that formed was collected by filtration, washed with water (10 mL) and dried under reduced pressure (high vacuum) to give **5.17a** (2.21 g, 91%) as a white solid.

mp 163-165°C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 2.87 (t, 2H, J = 6.6 Hz, C$\underline{H}_2$), 3.00 (d, 2H, J = 6.3 Hz, C$\underline{H}_2$), 7.61 (m, 3H, Ar$\underline{H}$), 7.85 (d, 1H, J = 7.8 Hz, Ar$\underline{H}$), 8.03 (dd, 2H, J = 2.9, 5.9 Hz, Ar$\underline{H}$), 8.30 (dd, 1H, J = 3.7, 7.6 Hz, Ar$\underline{H}$), 9.37 (s(br), 1H, CON$\underline{H}$), 10.89 (s(br), 1H, $CO_2\underline{H}$).

^{13}C NMR (acetone-d_6, 75 MHz) δ 29.2*, 31.1 ($\underline{C}H_2\underline{C}H_2$), 121.1, 122.9, 124.9, 125.6, 125.7, 125.9, 128.2, 134.2 (Ar$\underline{C}$), 170.9, 174.1 ($\underline{C}$ONH, $\underline{C}O_2$H).

FTIR (KBr, diffuse refraction method) cm^{-1} 1504.4, 1533.3, 1652.9, 1716.5, 2362.6, 2985.6, 3303.8.

HRMS (ES) 244.0977 (M^++H). $C_{14}H_{14}NO_3$ requires 244.0974.

Micro. Calcd for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.39; N, 5.76. Found: C, 69.20; H, 5.47; N, 6.02.

* Resonance at δ 29.2 ppm assigned from correlations observed in the HSQC and CIGAR NMR spectra.

Methyl *N*-(naphthalen-1-yl)succinamate (5.17b)

Acid **5.17a** (500 mg, 2.06 mmol, 1 equiv) was treated with diazomethane by General Procedure L. The crude product was purified by flash chromatography (ethyl acetate/petroleum ether, 2:1) to give **5.17b** (370 mg, 70%) as a white solid. An analytical sample was obtained by the diffusion of petroleum ether into a solution of **5.17b** dissolved in ethyl acetate.

mp 105-106°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.83 (s, 4H, $C\underline{H}_2C\underline{H}_2$), 3.74 (s, 3H, $CO_2C\underline{H}_3$), 7.44-7.55 (m, 3H, Ar$\underline{H}$), 7.68 (d, 1H, J = 8.3 Hz, Ar$\underline{H}$), 7.85-7.96 (m, 4H, Ar$\underline{H}$ plus CON$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 29.5, 32.0 ($\underline{C}H_2\underline{C}H_2$), 52.0 ($CO_2\underline{C}H_3$), 120.9, 125.6, 125.7, 125.9, 126.2, 127.1, 128.6, 132.3, 134.0 (Ar$\underline{C}$), 170.5, 173.8 ($\underline{C}$ONH, $\underline{C}O_2$Me).

FTIR (KBr, diffuse refraction method) cm^{-1} 1504.4, 1544.9, 1668.3, 1749.3, 3037.7, 3280.7.

HRMS (ES) 258.1136 (M^++H). $C_{15}H_{16}NO_3$ requires 258.1130.

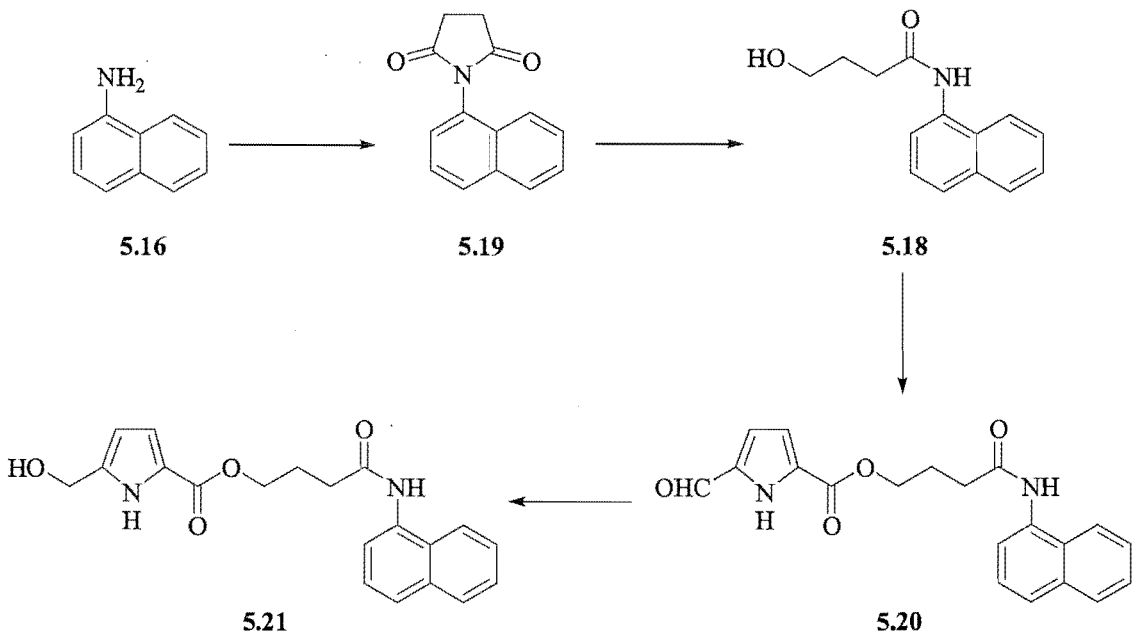
Micro. Calcd for $C_{15}H_{15}NO_3$: C, 70.02; H, 5.88; N, 5.44. Found: C, 70.06; H, 5.62; N, 5.69.

***N*-(Naphthalen-1-yl)succinamoyl chloride (5.17c)**

To a stirred suspension of the acid **5.17a** (300 mg, 1.23 mmol, 1 equiv) in dry dichloromethane (8 mL) at r.t. under an inert atmosphere were added oxalyl chloride (313 mg, 2.47 mmol, 2 equiv) and dry DMF (2 drops). Addition of the DMF briefly gave vigorous effervescence. After 1 h, the solvent was removed by evaporation under reduced

pressure to give a foaming solid. This product was used immediately without further purification or characterisation.

6.5.2 Preparation of the 5-(hydroxymethyl)pyrrole 5.21



***N*-(Naphthalen-1-yl)pyrrolidine-2,5-dione (5.19)**

Naphthalen-1-ylamine **5.16** (1.00 g, 6.98 mmol, 1 equiv) was treated with succinic anhydride (699 mg, 6.98 mmol, 1 equiv) by General Procedure P to give a white solid. The resulting material was treated with anhydrous sodium acetate (191 mg, 2.33 mmol, 0.33 equiv) according to General Procedure Q to give **5.19** (1.36 g, 86% over two steps) as a light pink solid.

mp 153-154°C (lit.[12] 147-149°C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.96-3.09 (m, 4H, C$\underline{H}_2$C$\underline{H}_2$), 7.33 (dd, 1H, J = 1.0, 7.3 Hz, Ar$\underline{H}$), 7.53 (m, 4H, Ar$\underline{H}$), 7.93 (m, 2H, Ar$\underline{H}$).

4-Hydroxy-*N*-(naphthalen-1-yl)butanamide (5.18)

The succinimide **5.19** (1.36 g, 6.04 mmol, 1 equiv) was treated with sodium borohydride (571 mg, 15.1 mmol, 2.5 equiv) by General Procedure R. After filtration through Dowex-

50W resin, the filtrate had its solvent removed by evaporation under reduced pressure. The crude product was purified by flash chromatography (ethyl acetate/methanol, 10:1), followed by recrystallisation from ethyl acetate, to give **5.18** (983 mg, 71%) as a white solid.

mp 102-104°C.

^{1}H NMR (CD_3OD, 500 MHz) δ 2.00 (m, 2H, $CH_2C\underline{H}_2CH_2$), 2.63 (t, 2H, J = 7.3 Hz, $C\underline{H}_2CO$), 3.70 (t, 2H, J = 6.3 Hz, $HOC\underline{H}_2$), 7.50 (m, 3H, Ar$\underline{H}$), 7.57 (d, 1H, J = 7.3 Hz, Ar$\underline{H}$), 7.77 (d, 1H, J = 8.3 Hz, Ar$\underline{H}$), 7.89 (m, 1H, Ar$\underline{H}$), 7.98 (d, 1H, J = 7.8 Hz, Ar$\underline{H}$).

^{13}C NMR (CD_3OD, 75 MHz) δ 30.0 ($CH_2\underline{C}H_2CH_2$), 34.2 ($\underline{C}H_2CO$), 62.6 ($HO\underline{C}H_2$), 123.7, 124.3, 126.7, 127.4, 127.5, 127.8, 129.6, 130.4, 134.4, 135.9 (Ar$\underline{C}$), 175.7 ($\underline{C}ONH$).

FTIR (KBr, diffuse refraction method) cm^{-1} 1508.2, 1541.0, 1652.9, 2950.9, 3274.9.

HRMS (EI) 229.1102 (M^+). $C_{14}H_{15}NO_2$ requires 229.1103.

Micro. Calcd for $C_{14}H_{15}NO_2$: C, 73.34; H, 6.59; N, 6.11. Found: C, 73.22; H, 6.68; N, 6.06.

4-(Naphthalen-1-ylamino)-4-oxobutyl 5-hydroxymethyl-1*H*-pyrrole-2-carboxylate (5.21)

Pyrrole acid **3.16b** (100 mg, 0.72 mmol, 1 equiv) was coupled to alcohol **5.18** (247 mg, 1.08 mmol, 1.5 equiv) by General Procedure S. The crude product was purified by flash chromatography (ethyl acetate/petroleum ether, 2:1) to give 4-(naphthalen-1-ylamino)-4-oxobutyl 5-formyl-1*H*-pyrrole-2-carboxylate (**5.20**, 95 mg, 0.27 mmol, 1 equiv) as an orange solid that contained trace impurities by ^{1}H NMR spectroscopy. The pyrrole **5.20** was reduced with lithium borohydride (12 mg, 0.54 mmol, 2 equiv) by General Procedure K. The crude product was purified by flash chromatography (ethyl acetate/petroleum ether, 5:1, then ethyl acetate), followed by recrystallisation from ethyl acetate, to give **5.21** (46 mg, 18% over two steps) as a white solid.

mp 154-156°C.

^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.15 (m, 2H, $CH_2C\underline{H}_2CH_2$), 2.74 (t, 2H, J = 7.3 Hz, $C\underline{H}_2CO$), 4.37 (t, 2H, J = 6.3 Hz, $CO_2C\underline{H}_2$), 4.51 (d, 2H, J = 5.4 Hz, $HOC\underline{H}_2$), 5.15 (t, 1H, J = 5.9 Hz, $\underline{H}OCH_2$), 6.17 (m, 1H, pyrrole $\underline{H}4$), 6.86 (s, 1H, pyrrole $\underline{H}3$), 7.60 (m, 3H,

ArH), 7.76 (d, 1H, J = 7.3 Hz, ArH), 7.85 (d, 1H, J = 8.3 Hz, ArH), 8.03 (m, 1H, ArH), 8.14 (m, 1H, ArH), 10.05 (s(br), 1H, CONH), 11.73 (s(br), 1H, pyrrole NH).

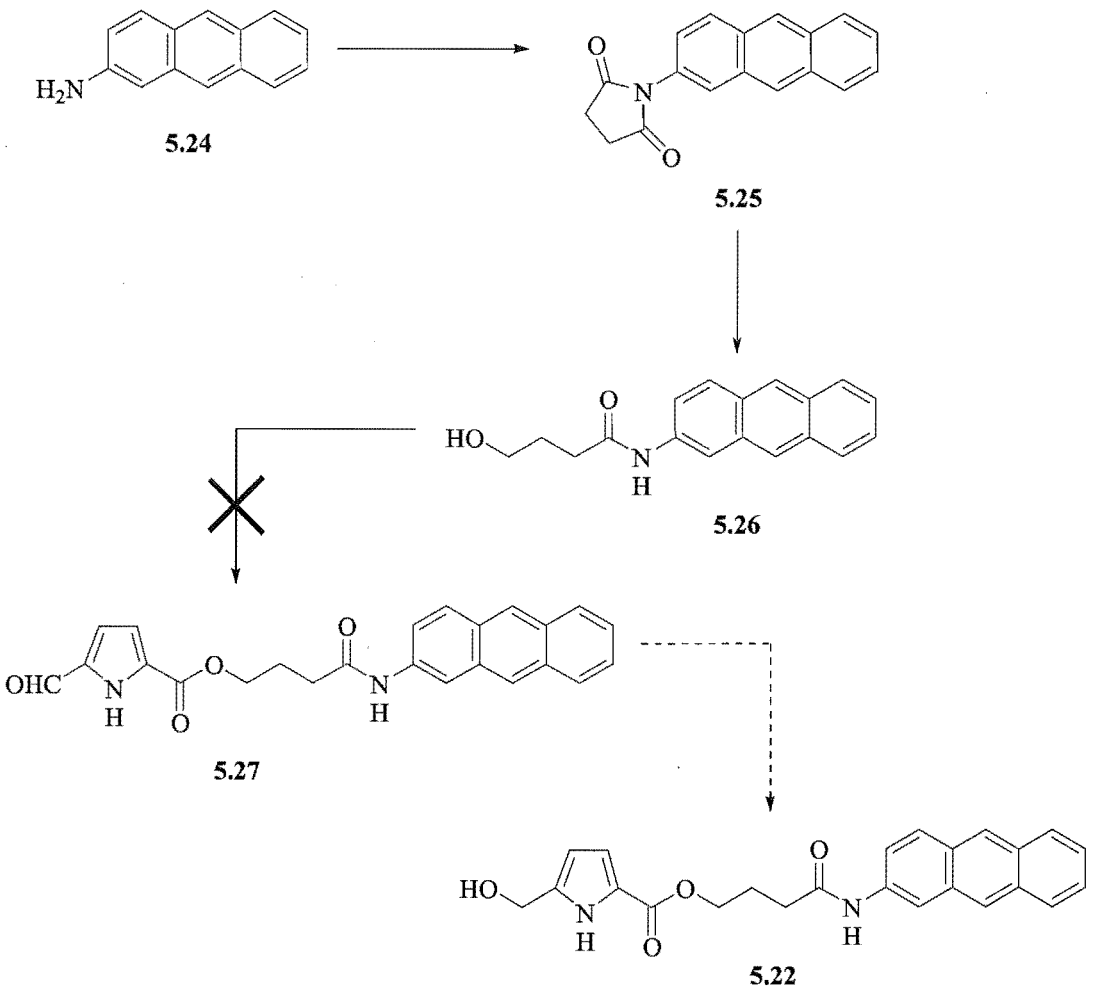
^{13}C NMR (DMSO-d_6, 75 MHz) δ 24.9 ($CH_2CH_2CH_2$), 32.5 (CH_2CO), 56.1 ($HOCH_2$), 63.1 (CO_2CH_2), 108.2 (pyrrole C4), 115.7 (pyrrole C3), 121.2 (pyrrole C2) 122.0, 122.9, 125.4, 125.8, 126.0, 126.2, 128.0, 128.3, 133.7, 133.9 (ArC), 139.4 (pyrrole C5), 160.7 (CO_2), 171.6 (CONH).

FTIR (KBr, diffuse refraction method) cm^{-1} 1500.5, 1531.4, 1654.8, 1685.7, 3282.6, 3433.1.

HRMS (ES) 375.1321 (M^++Na). $C_{20}H_{20}N_2NaO_4$ requires 375.1321.

Micro. Calcd for $C_{20}H_{20}N_2O_4$: C, 68.17; H, 5.72; N, 7.95. Found: C, 68.17; H, 5.79; N, 7.80.

6.5.5 Attempted preparation of the 5-(hydroxymethyl)pyrrole 5.22



***N*-(Anthracen-2-yl)pyrrolidine-2,5-dione (5.25)**

Anthracen-2-ylamine **5.24** (1.00 g, 5.17 mmol, 1 equiv) was treated with succinic anhydride (518 mg, 5.17 mmol, 1 equiv) by modified General Procedure P. The resulting material was treated with anhydrous sodium acetate (141 mg, 1.72 mmol, 0.33 equiv) by General Procedure Q to give **5.25** (1.27 g, 89% over two steps) as a brown solid.

mp 260-263°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.97 (s, 4H, $C\underline{H}_2C\underline{H}_2$), 7.35 (dd, 1H, J = 2.0, 8.8 Hz, Ar$\underline{H}$), 7.49 (m, 2H, Ar$\underline{H}$), 7.99 (m, 3H, Ar$\underline{H}$), 8.09 (d, 1H, J = 8.8 Hz, Ar$\underline{H}$), 8.45 (s, 2H, Ar$\underline{H}$).

^{13}C NMR (DMSO-d_6, 75 MHz) δ 28.8 ($\underline{C}H_2\underline{C}H_2$), 125.0, 126.1, 126.2, 126.4, 126.7, 128.2, 129.0, 130.0, 130.3, 130.7, 131.6, 131.8 (Ar$\underline{C}$), 177.4 (2 x $\underline{C}O_2$).

FTIR (KBr, diffuse refraction method) cm^{-1} 1581.5, 1624.0, 1716.5, 1770.5, 2947.0, 3051.2.

HRMS (EI) 275.0952 (M^+). $C_{18}H_{13}NO_2$ requires 275.0946.

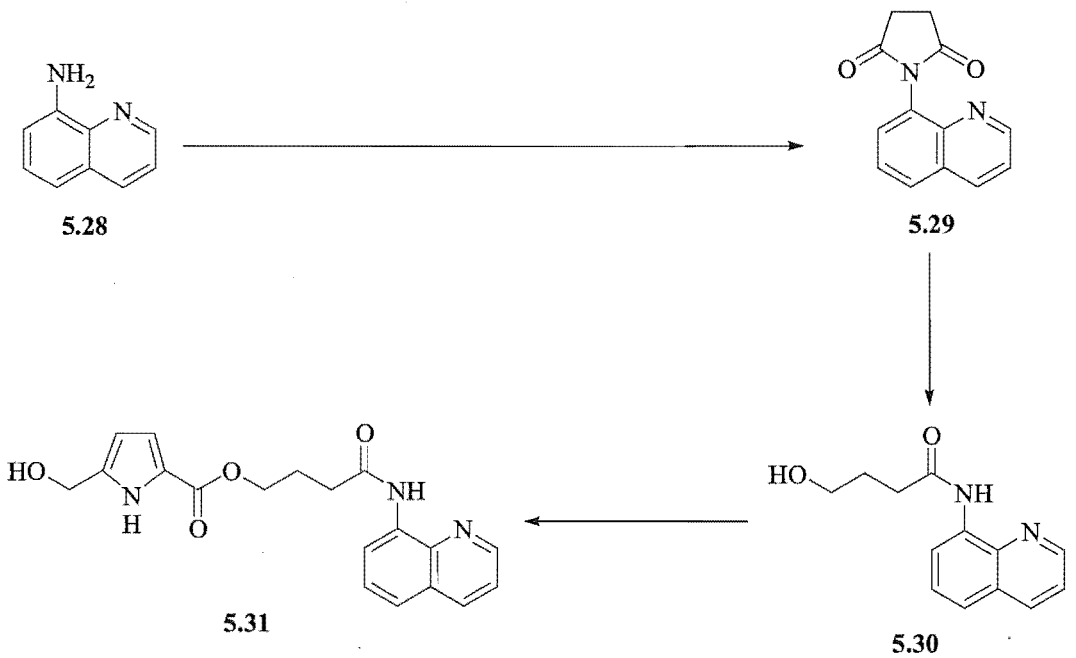
4-Hydroxy-*N*-(anthracen-2-yl)butanamide (5.26)

The succinimide **5.25** (370 mg, 1.34 mmol, 1 equiv) was treated with sodium borohydride (127 mg, 3.36 mmol, 2.5 equiv) by General Procedure R. The reaction mixture was acidified to pH ~1 by the dropwise addition of 1 M aqueous hydrochloric acid, and the *iso*-propanol was removed by rotary evaporation. The resulting solid was collected by filtration, washed with cold water and dried under reduced pressure (high vacuum) to give slightly impure **5.25** (375 mg, quantitative mass return) as a light brown solid. This product was used without further purification.

^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.89 (m, 2H, $CH_2C\underline{H}_2CH_2$), 2.54 (m, 2H, $C\underline{H}_2CO$), 7.56 (m, 2H, Ar$\underline{H}$), 7.65 (d, 1H, J = 7.8 Hz, Ar$\underline{H}$), 8.12 (m, 3H, Ar$\underline{H}$), 8.51 (s, 1H, Ar$\underline{H}$), 8.58 (m, 2H, Ar$\underline{H}$), 10.27 (s, 1H, CON$\underline{H}$).

The HOC$\underline{H}_2$ resonance was not observed due to the large $\underline{H}_2O$ signal present in the spectrum of **5.26**.

6.5.6 Preparation of the hydroxymethylpyrrole 5.31



***N*-(Quinolin-8-yl)pyrrolidine-2,5-dione (5.29)**

Quinolin-8-ylamine **5.28** (500 mg, 3.47 mmol, 1 equiv) was treated with succinic anhydride (347 mg, 3.47 mmol, 1 equiv) by General Procedure P. The resulting material was treated with sodium acetate (95 mg, 1.16 mmol, 0.33 equiv) by General Procedure Q to give **5.29** (620 mg, 79% over two steps) as a light brown solid.

mp 134-136°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.97, 3.13 (2 x m, 4H, C$\underline{H}_2$C$\underline{H}_2$), 7.44 (dd, 1H, J = 3.9, 8.3 Hz, Ar$\underline{H}$), 7.62 (m, 2H, Ar$\underline{H}$), 7.93 (dd, 1H, J = 2.0, 7.8 Hz, Ar$\underline{H}$), 8.20 (dd, 1H, J = 1.5, 9.8 Hz, Ar$\underline{H}$), 8.87 (dd, 1H, J = 1.7, 4.2 Hz, Ar$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 29.0 (C$\underline{H}_2$C$\underline{H}_2$), 122.0, 126.2, 129.3, 129.7, 129.8, 136.6, 150.8 (Ar$\underline{C}$), 176.9 (2 x $\underline{C}O_2$).

FTIR (KBr, diffuse refraction method) cm^{-1} 1502.4, 1701.1, 1778.2, 2947.0, 3051.2.

HRMS (EI) 226.0744 (M^+). $C_{13}H_{10}N_2O_2$ requires 226.0742.

4-(Quinolin-8-ylamino)-4-oxobutyl 5-hydroxymethyl-1*H*-pyrrole-2-carboxylate (5.31)

The succinimide **5.22** (496 mg, 2.19 mmol, 1 equiv) was treated with sodium borohydride (207 mg, 5.48 mmol, 2.5 equiv) by General Procedure R. After filtration through Dowex-50W resin, the filtrate had its solvent removed by evaporation under reduced pressure. The crude material was purified by flash chromatography (ethyl acetate, then ethyl acetate/methanol, 10:1) to give 4-hydroxy-*N*-(quinolin-8-yl)butanamide **(5.30**, 335 mg) as a sticky white solid.

^{1}H NMR (CD_3OD, 500 MHz) δ 1.99 (m, 2H, $CH_2C\underline{H}_2CH_2$), 2.68 (t, 2H, J = 7.6 Hz, $C\underline{H}_2CO$), 3.67 (t, 2H, J = 6.3 Hz, $HOC\underline{H}_2$), 7.53 (m, 2H, Ar$\underline{H}$), 7.61 (m, 1H, Ar$\underline{H}$), 8.29 (dd, 1H, J = 2.0, 8.3 Hz, Ar$\underline{H}$), 8.62 (d, 1H, J = 7.8 Hz, Ar$\underline{H}$), 8.86 (d, 1H, J = 1.5, 4.4 Hz, Ar$\underline{H}$).

Pyrrole acid **3.16b** (135 mg, 0.97 mmol, 1 equiv) was coupled to the alcohol (335 mg, 1.45 mmol, 1.5 equiv) by General Procedure S. The crude material was purified by flash chromatography (ethyl acetate/petroleum ether, 2:1) to give 4-(quinolin-8-ylamino)-4-oxobutyl 5-formyl-1*H*-pyrrole-2-carboxylate (150 mg, 0.43 mmol, 1 equiv) as an orange solid that contained trace impurities by ^{1}H NMR spectroscopy. This pyrrole was reduced with lithium borohydride (19 mg, 0.85 mmol, 2 equiv) by General Procedure K. The crude material was purified by flash chromatography (ethyl acetate/petroleum ether, 4:1, then ethyl acetate), followed by recrystallisation from ethyl acetate/petroleum ether, to give **5.31** (86 mg, 11% over three steps) as a tan solid.

mp 118-119°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.25 (m, 2H, $CH_2C\underline{H}_2CH_2$), 2.57 (s(br), 1H, O$\underline{H}$), 2.71 (t, 2H, J = 7.1 Hz, $C\underline{H}_2CO$), 4.39 (t, 2H, J = 6.3 Hz, $CO_2C\underline{H}_2$), 4.63 (s, 2H, $HOC\underline{H}_2$), 6.17 (t, 1H, J = 3.2 Hz, pyrrole $\underline{H}4$), 6.83 (dd, 1H, J = 2.4, 3.4 Hz, pyrrole $\underline{H}3$), 7.43 (dd, 1H, J = 4.4, 8.3 Hz, Ar$\underline{H}$), 7.51 (m, 2H, Ar$\underline{H}$), 8.14 (dd, 1H, J = 1.7, 8.1 Hz, Ar$\underline{H}$), 8.74 (m, 2H, Ar$\underline{H}$), 9.77, 9.85 (2 x s(br), 2H, CON$\underline{H}$ and pyrrole N$\underline{H}$).

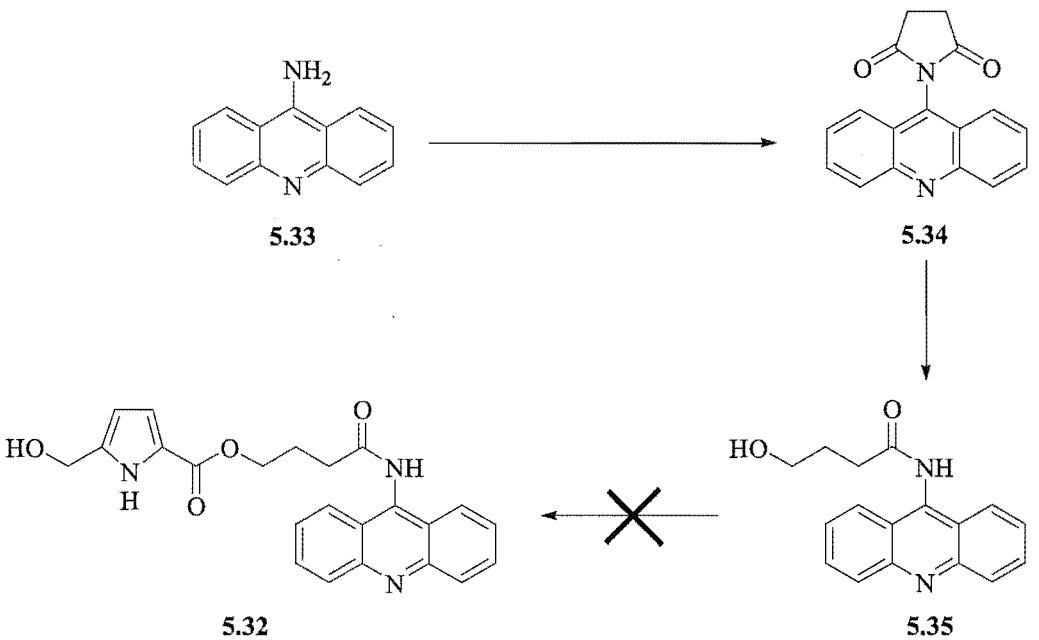
^{13}C NMR ($CDCl_3$, 75 MHz) δ 24.2 ($CH_2\underline{C}H_2CH_2$), 33.7 ($\underline{C}H_2CO$), 56.7 ($HO\underline{C}H_2$), 62.5 ($CO_2\underline{C}H_2$), 107.2 (pyrrole $\underline{C}4$), 114.9 (pyrrole $\underline{C}3$), 116.3, 121.2, 126.8, 127.4, 133.6, 136.4, 137.2, 147.4 (Ar$\underline{C}$), 121.4 (pyrrole $\underline{C}2$), 137.8 (pyrrole $\underline{C}5$), 160.5 ($\underline{C}O_2$), 170.3 ($\underline{C}ONH$).

FTIR (KBr, diffuse refraction method) cm^{-1} 1539.1, 1652.9, 1708.8, 2949.0, 3261.4, 3348.2.

HRMS (EI) 353.1362 (M^+). $C_{19}H_{19}N_3O_4$ requires 353.1376.

Micro. Calcd for $C_{19}H_{19}N_3O_4$: C, 64.58; H, 5.42; N, 11.89. Found: C, 64.46; H, 5.59; N, 11.65.

6.5.7 Attempted preparation of the 5-(hydroxymethyl)pyrrole 5.32



***N*-(Acridin-9-yl)pyrrolidine-2,5-dione (5.34)**

A stirred solution of acridin-9-ylamine **5.33** (600 mg, 3.09 mmol, 1 equiv) and succinic anhydride (309 mg, 3.09 mmol, 1 equiv) in dry DMF (15 mL), under an inert atmosphere, was refluxed for 30 min. After cooling to r.t., the solution was poured onto ice (10 g) and water (10 mL), and the resultant solid was allowed to stand for 3 h, after which it was collected by filtration, washed with water, and dried under reduced pressure (high pressure). This material was treated with anhydrous sodium acetate (84 mg, 1.03 mmol, 0.33 equiv) by General Procedure Q to give **5.34** (301 mg, 35% over two steps) as a light brown solid.

mp 269-272°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.24 (s, 4H, $C\underline{H}_2C\underline{H}_2$), 7.60 (m, 2H, Ar$\underline{H}$), 7.67 (d, 2H, J = 8.3 Hz, Ar$\underline{H}$), 7.82 (m, 2H, Ar$\underline{H}$), 8.34 (d, 2H, J = 8.8 Hz, Ar$\underline{H}$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 29.1 ($\underline{C}H_2\underline{C}H_2$), 122.1, 127.6, 130.2, 130.6, 149.1 (Ar$\underline{C}$), 175.7 (2 x $\underline{C}O_2$).

FTIR (KBr, diffuse refraction method) cm^{-1} 1517.9, 1554.5, 1710.7, 1786.0, 2937.4, 3058.9.

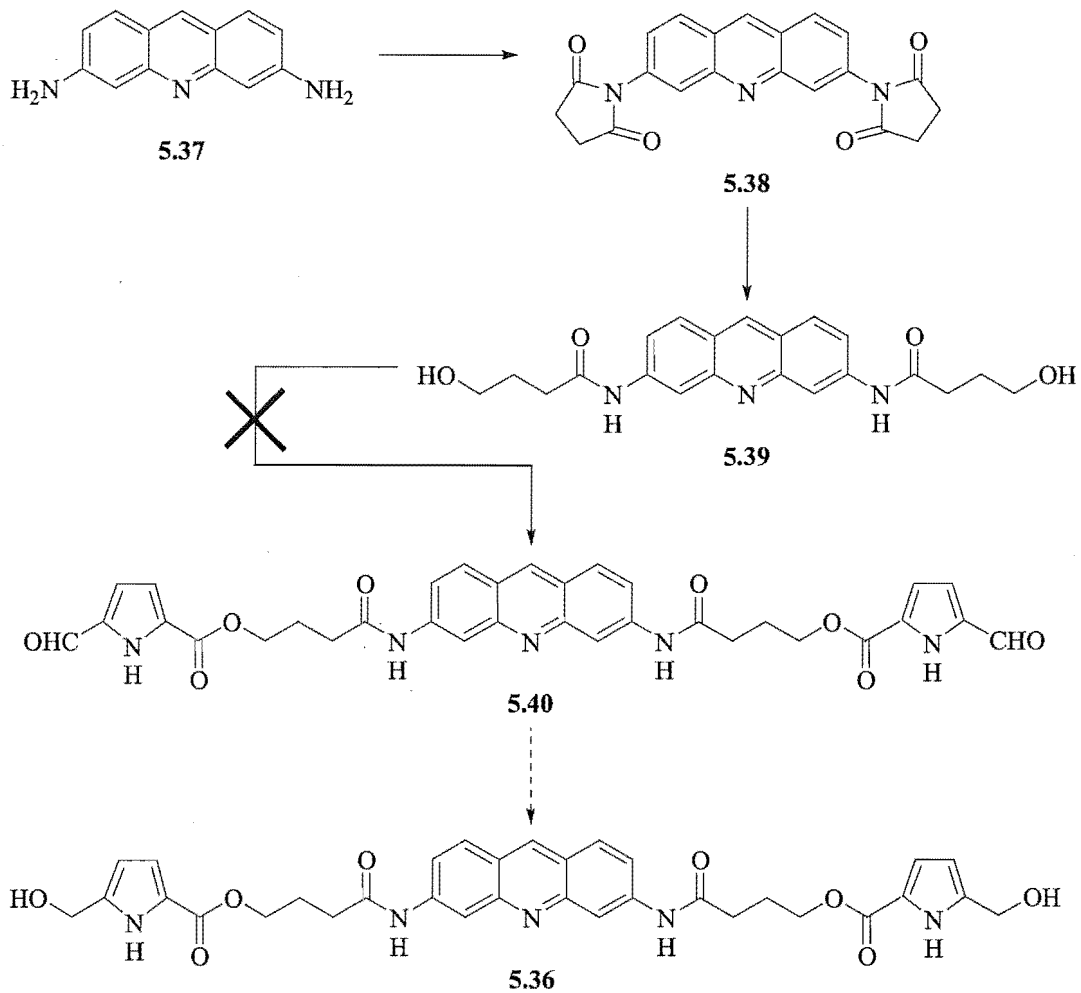
HRMS (EI) 276.0907 (M^+). $C_{17}H_{12}N_2O_2$ requires 276.0899.

4-Hydroxy-*N*-(acridin-9-yl)butanamide (5.35)

The succinimide **5.34** (500 mg, 1.81 mmol, 1 equiv) was treated with sodium borohydride (171 mg, 4.52 mmol, 2.5 equiv) by General Procedure R. After filtration through Dowex-50W resin, the resin was stirred in methanol for 30 min and filtered once again, the filtrates were combined, and the solvent was removed by evaporation under reduced pressure. The resulting material was passed through a short plug of silica (eluted with methanol) to give impure **5.35** (449 mg, 89% mass return) as a yellow solid. This product was used without further purification.

^{1}H NMR (CD_3OD, 500 MHz) δ 2.08 (m, 2H, $CH_2C\underline{H}_2CH_2$), 2.84 (t, 2H, J = 7.6 Hz, $C\underline{H}_2CO$), 3.76 (t, 2H, J = 6.3 Hz, $HOC\underline{H}_2$), 7.64 (m, 2H, Ar$\underline{H}$), 7.86 (m, 2H, Ar$\underline{H}$), 8.18 (m, 4H, Ar$\underline{H}$).

6.5.8 Attempted preparation of the hydroxymethylpyrrole 5.36



***N,N'*-(Acridin-3,6-diyl)dipyrrolidine-2,5-dione (5.38)**

A stirred suspension of 3,6-diaminoacridine **5.37** (1.84 g, 8.79 mmol, 1.5 equiv) and succinic anhydride (1.94 g, 19.3 mmol, 3.3 equiv) in dry pyridine (20 mL), under an inert atmosphere, was refluxed for 2 h. After cooling to r.t., the solution was poured onto ice (20 g) and water (20 mL), and the resulting solid was allowed to stand for 2 h, after which it was collected by filtration, washed with cold water and dried under reduced pressure (high vacuum). This material was suspended in dry acetic anhydride (25 mL) under an inert atmosphere at r.t., to which was added anhydrous sodium acetate (481 mg, 5.86 mmol, 1 equiv). Workup was performed as for General Procedure Q. The crude product was purified by flash chromatography on silica (ethyl acetate/acetone 2:1, then ethyl

acetate/acetone 1:2, then acetone), followed by recrystallisation (ethyl acetate/petroleum ether), to give **5.38** (169 mg, 5% yield over two steps) as an orange solid.

mp 337-339°C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 3.09 (s, 8H, 2 x C$\underline{H}_2$C$\underline{H}_2$), 7.72 (dd, 2H, J = 2.0, 8.8 Hz, Ar$\underline{H}$), 8.29 (t, 2H, J = 1.0 Hz, Ar$\underline{H}$), 8.37 (d, 2H, J = 9.3 Hz, Ar$\underline{H}$), 9.26 (s, 1H, Ar$\underline{H}$).

^{13}C NMR (DMSO-d_6, 75 MHz) δ 28.8 (2 x $\underline{C}$H$_2\underline{C}$H$_2$), 125.3, 125.5, 126.4, 129.3, 134.9, 136.7, 148.4 (Ar$\underline{C}$), 177.0 (4 x $\underline{C}$O$_2$).

FTIR (KBr, diffuse refraction method) cm^{-1} 1560.3, 1624.0, 1701.71 1782.1, 2947.0, 2991.4.

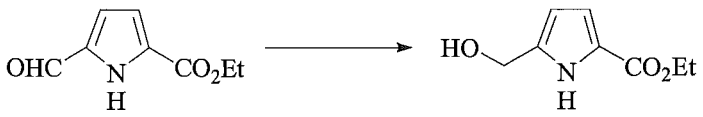
HRMS (EI) 373.1068 (M^+). $C_{21}H_{15}N_3O_4$ requires 373.1063.

4,4'-Hydroxy-*N*,*N*'-(acridin-3,6-diyl)dibutanamide (5.39)

The succinimide **5.38** (160 mg, 0.43 mmol, 1 equiv) was treated with sodium borohydride (171 mg, 4.52 mmol, 5 equiv) by modified General Procedure R. After filtration through Dowex-50W resin, the resin was stirred in methanol for 30 min and filtered once again, the filtrates were combined, and the solvent was removed by evaporation under reduced pressure. The resulting material was passed through a short plug of silica (eluted with methanol) to give **5.39** (449 mg, 89%) as a brown solid. This product was used without further purification.

^{1}H NMR (CD_3OD, 500 MHz) δ 1.89 (m, 4H, 2 x CH$_2$C$\underline{H}_2$CH$_2$), 2.42 (t, 4H, J = 7.3 Hz, 2 x C$\underline{H}_2$CO), 3.61 (t, 4H, J = 6.3 Hz, 2 x HOC$\underline{H}_2$), 6.81 (dd, 2H, J = 2.0, 8.3 Hz, Ar$\underline{H}$), 6.94 (m, 3H, Ar$\underline{H}$), 7.11 (d, 2H, J = 2.0 Hz, Ar$\underline{H}$).

6.5.9 Preparation of the 5-(hydroxymethyl)pyrrole 5.41



3.16a **5.41**

Ethyl 5-hydroxymethyl-1*H*-pyrrole-2-carboxylate (5.41)

Pyrrole ester **3.16a** (150 mg, 0.90 mmol, 1 equiv) was reduced with lithium borohydride (39 mg, 1.79 mmol, 2 equiv) by General Procedure K. The crude product was purified by flash chromatography on silica (ethyl acetate/petroleum ether, 1:1) to give **5.41** (115 mg, 76%) as a white solid.

mp 76-78°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.35 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$), 4.31 (q, 2H, J = 7.2 Hz, $C\underline{H}_2CH_3$), 4.69 (s, 2H, $HOC\underline{H}_2$), 6.11 (t, 1H, J = 3.2 Hz, pyrrole H4), 6.84 (dd, 1H, J = 2.4, 3.9 Hz, pyrrole H3), 9.51 (s(br), 1H, pyrrole NH).

^{13}C NMR (Acetone-d_6, 75 MHz) δ 14.1 ($CH_2\underline{C}H_3$), 57.0 ($HO\underline{C}H_2$), 59.6 ($\underline{C}H_2CH_3$), 108.0 (pyrrole C4), 115.2 (pyrrole C3), 122.4 (pyrrole C2), 138.7 (pyrrole C5), 160.7 ($\underline{C}O_2$).

FTIR (KBr, diffuse refraction method) 1502.4, 1683.7, 2993.3, 3286.5.

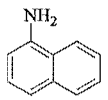
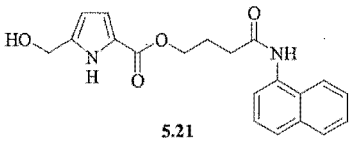
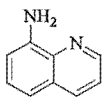
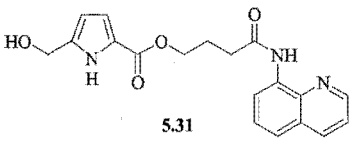
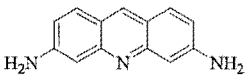
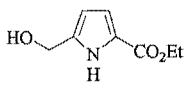
HRMS (EI) 169.0739 (M^+). $C_8H_{11}NO_3$ requires 169.0738.

Micro. Calcd for $C_8H_{11}NO_3$: C, 56.80; H, 6.55; N, 8.28. Found: C, 57.03; H, 6.63; N, 8.19.

6.5.10 Biological Assays

P388 Biological Assay[28]

This is an antitumour assay, whereby a dilution series of a prepared compound in methanol or 3:1 methanol/DMF with P388 murine leukaemia cells is incubated for 72 h. The concentration of the sample required to reduce the P388 cell growth by 50%, compared to the control cells, is determined using the absorbance values obtained at 540 nm when the yellow MTT tetrazolium is reduced by healthy cells to produce purple coloured MTT formazan. The result is expressed as an ID_{50} in μg mL^{-1} and μM.

No.	Solvent system*	Sample concentration (mg mL^{-1})#	ID_{50} (μg mL^{-1})	ID_{50} (μM)
 5.16	A	10	87	611
 5.21	B	20	165	468
 5.28	A	10	13	88
 5.31	A	10	111	314
 5.37	B	1	2.0	9.4
 5.41	A	10	>125	>739

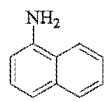
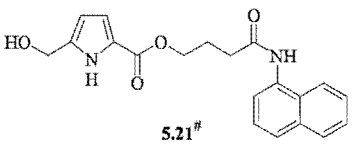
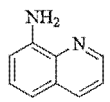
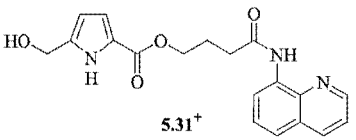
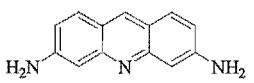
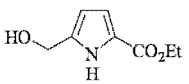
* A = MeOH, B = 3:1 MeOH/DMF
# Concentration of compound submitted for analysis

Table 6.6. Activity of **5.16, 5.21, 5.28, 5.31, 5.37** and **5.41** against the P388 murine leukaemia cell line.

Antiviral Assay

Aliquots, typically 40 μL, of a prepared compound are pipetted onto 6 mm diameter filter paper discs. The solvent is allowed to evaporate before the discs are placed directly onto BSC-1 cells (African Green Monkey Kidney), infected with either Herpes simplex type 1 virus (ATCC VR 733), or Polio virus type 1 (Pfizer vaccine strain). After a 24 h incubation period at 37°C in an atmosphere containing 1.5% carbon dioxide the wells are

examined using an inverted microscope. The assay is scored based on the size of viral inhibition and/or cytotoxic zones, and the type of cytotoxicity.

No.	PV 1		HSV 1	
	Antiviral effects	Cytotoxic effects	Antiviral effects	Cytotoxic effects
 **5.16**$^{+}$	?	4+	?	4+
 **5.21**$^{\#}$	ND	ND	ND	ND
 **5.28**$^{+}$	?*	2+	?*	2+
 **5.31**$^{+}$	?	4+	?*	3+
 **5.37**$^{+}$	?*	+	?*	2+
 **5.41**$^{+}$	ND	ND	ND	ND

$^{+}$ Activity tested in the solvent system and sample concentration specified in Table 5.1.
$^{\#}$ Activity tested in solvent system A at 10 mg mL^{-1}.

Table 6.7. Activity of **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41** against the Polio type 1 virus (PV 1) and Herpes simplex type 1 virus (HSV 1).

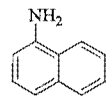
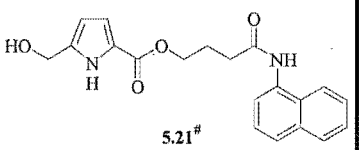
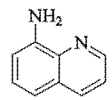
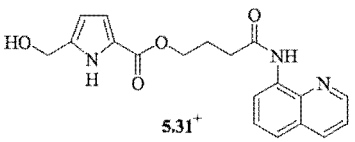
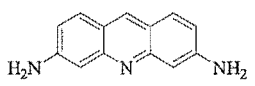
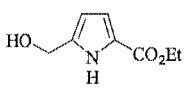
Antimicrobial Assay

Bacteria or fungi at a known concentration are mixed with a potato dextrose agar and poured into petri dishes so that after incubation a "lawn" of bacteria/fungi will grow over the entire dish. Samples of interest are pipetted onto 6 mm diameter paper discs and their solvents evaporated. These are then placed onto the above prepared seeded agar dishes (with appropriate solvent and positive controls) and incubated. Zones of inhibition are measured in millimetres as the radius from the centre of the disc to the edge of the inhibited zone. There are six test organisms:

Bacteria: *Escherichia coli* (*E coli*), *Bacillus subtilis* (*B sub*)*, *Pseudomonas aeruginosa* (*P aer*)

Fungi: *Candida albicans* (*C alb*), *Trichophyton mentagrophytes* (*T ment*)*, *Cladosporium resinae* (*C res*)

* = most sensitive organisms.

No.	Bacteria			Fungi		
	E coli	*B sub*	*P aer*	*C alb*	*T ment*	*C res*
 **5.16**$^{+}$	4	3	1	10	22	10
 **5.21**$^{\#}$	-¶	-	-	-	-	-
 **5.28**$^{+}$	3	10	-	15	10	15
 **5.31**$^{+}$	-	-	-	-	1	-
 **5.37**$^{+}$	2	10	1	1	6	3
 **5.41**$^{+}$	-	-	-	-	3	-
Gentamycin	10		10			
Chloramphenicol		13				
Nystatin				10	7	12

$^{+}$ Activity tested in the solvent system and sample concentration specified in Table 5.1.
$^{\#}$ Activity tested in solvent system A at 10 mg mL^{-1}.
¶No activity observed.

Table 6.8. Activity of **5.16**, **5.21**, **5.28**, **5.31**, **5.37** and **5.41** against selected microbial species.

6.6 REFERENCES FOR CHAPTER SIX

1. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

2. Armarego, W. L. F.; Perrin, D. D. *Purification of Laboratory Chemicals*; 4th ed.; Butterworth-Heinemann: Oxford, 1996.

3. Budesinsky, Z.; Sluka, J.; Novak, J.; Danek, J. *Coll. Czech. Chem. Commun.* **1975**, *40*, 1089.

4. Alcalde, E.; Dinares, I.; Perez-Garcia, L.; Roca, T. *Synthesis* **1992**, 395.

5. Dodd, J. H. In *Encyclopedia of Reagents for Organic Synthesis*; Paquette, L. A., Ed.; John Wiley & Sons Ltd.: Chichester, 1995; Vol. 6, pp. 4169-4175.

6. Rangarajan, M.; Kim, J. S.; Sim, S.-P.; Liu, A.; Liu, L. F.; LaVoie, E. J. *Bioorg. Med. Chem.* **2000**, *8*, 2591.

7. Tani, M.; Ariyasu, T.; Nishiyama, C.; Hagiwara, H.; Watanabe, T.; Yokoyama, Y.; Murakami, Y. *Chem. Pharm. Bull.* **1996**, *44*, 48.

8. Bailey, D. M.; Johnson, R. E.; Albertson, N. F. *Org. Synth.* **1971**, *51*, 100.

9. Tian, Z.-Q.; Brown, B. B.; Mack, D. P.; Hutton, C. A.; Bartlett, P. A. *J. Org. Chem.* **1997**, *62*, 514.

10. Abell, A. D.; Nabbs, B. K.; Battersby, A. R. *J. Am. Chem. Soc.* **1998**, *120*, 1741.

11. Le Nguyen, D.; Castro, B. *Peptide Chemistry 1987*; Protein Research Foundation: Osaka, 1988.

12. Hubbard, J. L.; Carl III, J. M.; Anderson, G. D. *J. Heterocyclic Chem.* **1992**, *29*, 720.

13. Beckwith, A. L. J.; Boate, D. R. *J. Org. Chem.* **1988**, *53*, 4339.

14. Defacqz, N.; Touillaux, R.; Cordi, A.; Marchand-Brynaert, J. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2632.

15. Diago-Meseguer, J.; Palomo-Coll, A. L.; Fernandez-Lizarbe, J. R.; Zugaza-Bilbao, A. *Synthesis* **1980**, 547.

16. Muathen, H. A. *J. Chem. Res. (M)* **1994**, 2201.

17. Itaya, M. *Yakugaku Zasshi* **1962**, *82*, 1.

18. Shine, H. J.; Stanley, J. P. *J. Org. Chem.* **1967**, *32*, 905.

19. Sus, O.; Moller, K.; Heiss, H. *Justus Liebigs Ann. Chem.* **1956**, *598*, 123.

20. Campbell, N.; Anderson, W.; Gilmore, J. *J. Chem. Soc.* **1940**, 446.

21. Atkinson, C. M.; Sharpe, C. J. *J. Chem. Soc.* **1959**, 2858.

22. Ichikawa, M.; Nabeya, S.; Muraoka, K.; Hisano, T. *Chem. Pharm. Bull.* **1979**, *27*, 1255.

23. Cannell, R. J. P.; Kellam, S. J.; Owsianka, A. M.; Walker, J. M. *Planta Med.* **1988**, *54*, 10.

24. Khan, M. K. A.; Morgan, K. J.; Morrey, D. P. *Tetrahedron* **1966**, *22*, 2095.

25. Kessler, H.; Muller, A. *Liebigs Ann. Chem.* **1986**, 1687.

26. Noss, L.; Liddell, P. A.; Moore, A. L.; Moore, T. A.; Gust, D. *J. Phys. Chem. B* **1997**, *101*, 458.

27. Nabbs, B. K., Ph.D. thesis, University of Canterbury, 1999.

28. Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 589

APPENDIX

CRYSTALLOGRAPHIC STRUCTURE DETERMINATION FOR COMPOUND 4.11h BY X-RAY ANALYSIS

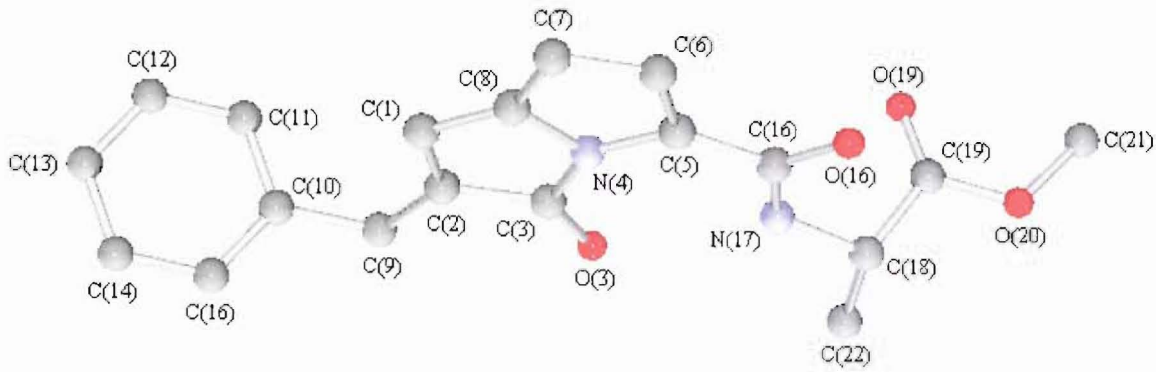


Figure A1. Labelled drawing of **4.11h**.

Instrument	Siemens P4 four circle diffractometer	
Empirical formula	$C_{19}H_{18}N_2O_4$	
Molecular weight	338.35 g mol^{-1}	
Colour and shape	Orange block	
Temperature	168(2) K	
Wavelength	0.71073 Å (Mo-Kα radiation)	
Crystal system	Orthorhombic	
Space group	*I*222	
Unit cell dimensions	a = 7.880(4) Å	α = 90°.
	b = 13.748(5) Å	β = 90°.
	c = 34.669(16) Å	γ = 90°.
Crystal size	0.80 x 0.25 x 0.25 mm	
Volume	3756(3) $Å^3$	
Z	8	
Density (calculated)	1.197 g cm^{-3}	
Absorption coefficient	0.085 mm^{-1}	
F(000)	1424	
θ range for data collection	2.65 to 26.31°.	

Index ranges	$-9 \leq h \leq 9, -6 \leq k \leq 15, -42 \leq l \leq 43$
Reflections collected	7633
Independent reflections	3570 [$R_{(int)} = 0.0257$]
Completeness to theta = 26.31°	93.3 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9791 and 0.9351
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	3570/0/228
Goodness-of-fit on F^2	1.070
Final R indices [$I > 2\sigma(I)$]	$R_1 = 0.0496$, $wR_2 = 0.1506$
R indices (all data)	$R_1 = 0.0574$, $wR_2 = 0.1577$
Absolute structure parameter	1.1(14)
Largest diff. peak and hole	0.551 and -0.266 eÅ^{-3}

The unique reflections were used in a full-matrix least-squares refinement.[1] The structure was solved by direct methods.[2] Hydrogen atoms were fixed in idealised positions. All non-hydrogen atoms were refined with anisotropic displacement parameters.

Atom	x	y	z	U(eq)
C(1)	10483(3)	1261(2)	8245(1)	34(1)
C(2)	8839(3)	1234(2)	8146(1)	31(1)
C(3)	8713(3)	1243(2)	7714(1)	29(1)
C(5)	11376(3)	1254(2)	7239(1)	31(1)
C(6)	13044(3)	1268(2)	7347(1)	37(1)
C(7)	13136(3)	1281(2)	7760(1)	40(1)
C(8)	11505(3)	1272(2)	7895(1)	34(1)
C(9)	7266(3)	1202(2)	8393(1)	35(1)
C(10)	7629(3)	1207(2)	8820(1)	35(1)
C(11)	8258(4)	2038(2)	8996(1)	47(1)
C(12)	8600(4)	2053(3)	9388(1)	59(1)
C(13)	8298(4)	1237(3)	9610(1)	61(1)
C(14)	7675(5)	411(3)	9436(1)	65(1)
C(15)	7341(4)	394(2)	9045(1)	50(1)
C(16)	10737(3)	1227(2)	6837(1)	32(1)
C(18)	8342(3)	1177(2)	6398(1)	36(1)
C(19)	8630(3)	2105(2)	6172(1)	35(1)
C(21)	8719(5)	2736(3)	5540(1)	67(1)
C(22)	6436(4)	1001(2)	6432(1)	52(1)
N(4)	10409(2)	1260(1)	7581(1)	30(1)
N(17)	9047(2)	1247(2)	6784(1)	35(1)
O(3)	7443(2)	1232(1)	7516(1)	37(1)
O(16)	11749(2)	1194(1)	6567(1)	40(1)
O(19)	8723(2)	2910(1)	6302(1)	41(1)
O(20)	8652(3)	1896(1)	5793(1)	53(1)

Table A1. Atomic coordinates (x 10^{-4}) and equivalent isotropic displacement parameters ($Å^2$ x 10^{-3}) for **4.11h**. U(eq) is defined as one third of the trace of the orthogonalized U_{ij} tensor.

Bond	Length (Å)	Bond	Length (Å)	Bond	Length (Å)
C(1)-C(2)	1.341(3)	C(6)-C(7)	1.433(4)	C(14)-C(15)	1.380(4)
C(1)-C(8)	1.455(3)	C(7)-C(8)	1.369(3)	C(16)-O(16)	1.231(3)
C(2)-C(3)	1.502(3)	C(8)-N(4)	1.390(3)	C(16)-N(17)	1.344(3)
C(2)-C(9)	1.506(3)	C(9)-C(10)	1.509(3)	C(18)-N(17)	1.454(3)
C(3)-O(3)	1.214(3)	C(10)-C(15)	1.382(4)	C(18)-C(19)	1.515(3)
C(3)-N(4)	1.414(3)	C(10)-C(11)	1.387(4)	C(18)-C(22)	1.526(4)
C(5)-C(6)	1.366(3)	C(11)-C(12)	1.385(4)	C(19)-O(19)	1.198(3)
C(5)-N(4)	1.410(3)	C(12)-C(13)	1.380(5)	C(19)-O(20)	1.344(3)
C(5)-C(16)	1.483(3)	C(13)-C(14)	1.376(5)	C(21)-O(20)	1.450(3)

Table A2. Bond lengths (Å) for **4.11h**.

Bond	Angle (°)	Bond	Angle (°)
C(2)-C(1)-C(8)	108.80(18)	C(12)-C(11)-C(10)	120.9(3)
C(1)-C(2)-C(3)	108.56(18)	C(13)-C(12)-C(11)	120.1(3)
C(1)-C(2)-C(9)	130.60(19)	C(14)-C(13)-C(12)	119.2(3)
C(3)-C(2)-C(9)	120.84(18)	C(13)-C(14)-C(15)	120.8(3)
O(3)-C(3)-N(4)	126.55(18)	C(14)-C(15)-C(10)	120.6(3)
O(3)-C(3)-C(2)	128.2(2)	O(16)-C(16)-N(17)	122.6(2)
N(4)-C(3)-C(2)	105.23(17)	O(16)-C(16)-C(5)	119.7(2)
C(6)-C(5)-N(4)	106.77(18)	N(17)-C(16)-C(5)	117.61(19)
C(6)-C(5)-C(16)	125.8(2)	N(17)-C(18)-C(19)	111.38(19)
N(4)-C(5)-C(16)	127.41(19)	N(17)-C(18)-C(22)	108.3(2)
C(5)-C(6)-C(7)	108.9(2)	C(19)-C(18)-C(22)	108.7(2)
C(8)-C(7)-C(6)	107.1(2)	O(19)-C(19)-O(20)	124.5(2)
C(7)-C(8)-N(4)	108.32(19)	O(19)-C(19)-C(18)	126.2(2)
C(7)-C(8)-C(1)	143.7(2)	O(20)-C(19)-C(18)	109.1(2)
N(4)-C(8)-C(1)	108.02(19)	C(8)-N(4)-C(5)	108.91(17)
C(2)-C(9)-C(10)	113.68(18)	C(8)-N(4)-C(3)	109.38(17)
C(15)-C(10)-C(11)	118.4(2)	C(5)-N(4)-C(3)	141.70(17)
C(15)-C(10)-C(9)	121.3(2)	C(16)-N(17)-C(18)	120.13(19)
C(11)-C(10)-C(9)	120.2(2)	C(19)-O(20)-C(21)	114.8(2)

Table A3. Bond angles (°) for **4.11h**.

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
C(1)	35(1)	37(1)	29(1)	2(1)	-3(1)	4(1)
C(2)	34(1)	29(1)	29(1)	2(1)	0(1)	0(1)
C(3)	32(1)	25(1)	31(1)	2(1)	0(1)	2(1)
C(5)	33(1)	27(1)	33(1)	-1(1)	2(1)	-4(1)
C(6)	32(1)	37(1)	42(1)	1(1)	2(1)	2(1)
C(7)	31(1)	50(1)	39(1)	0(1)	-6(1)	-1(1)
C(8)	32(1)	37(1)	32(1)	-1(1)	-6(1)	2(1)
C(9)	32(1)	42(1)	32(1)	1(1)	-2(1)	0(1)
C(10)	32(1)	44(1)	30(1)	4(1)	2(1)	6(1)
C(11)	50(2)	56(2)	35(1)	4(1)	0(1)	-8(1)
C(12)	52(2)	84(2)	41(2)	-15(1)	-3(1)	-6(2)
C(13)	65(2)	88(2)	31(1)	6(2)	-2(1)	15(2)
C(14)	79(2)	74(2)	43(2)	24(2)	6(2)	21(2)
C(15)	59(2)	49(2)	42(1)	5(1)	9(1)	9(1)
C(16)	37(1)	25(1)	33(1)	4(1)	-1(1)	-1(1)
C(18)	41(1)	35(1)	33(1)	2(1)	-4(1)	-4(1)
C(19)	30(1)	46(1)	30(1)	2(1)	-1(1)	0(1)
C(21)	88(2)	77(2)	37(1)	22(1)	1(2)	-11(2)
C(22)	46(2)	58(2)	51(2)	17(1)	-15(1)	-15(1)
N(4)	28(1)	35(1)	28(1)	1(1)	-3(1)	-1(1)
N(17)	32(1)	43(1)	29(1)	4(1)	-1(1)	-2(1)
O(3)	29(1)	51(1)	32(1)	4(1)	-4(1)	-2(1)
O(16)	40(1)	45(1)	34(1)	0(1)	6(1)	-3(1)
O(19)	42(1)	37(1)	43(1)	1(1)	1(1)	-3(1)
O(20)	75(1)	56(1)	28(1)	2(1)	0(1)	-6(1)

Table A4. Anisotropic displacement parameters ($Å^2$ x 10^{-3}) for **4.11h**. The anisotropic displacement factor exponent takes the form: $-2p^2[(ha^*)^2U^{11} + ... + 2hka^*b^*U^{12}]$.

Atom	x	y	z	U(eq)
H(1)	10911	1270	8501	40
H(6)	13987	1269	7177	44
H(7)	14141	1293	7911	48
H(9A)	6615	608	8329	42
H(9B)	6546	1771	8330	42
H(11)	8457	2604	8846	56
H(12)	9042	2625	9504	71
H(13)	8519	1246	9879	74
H(14)	7471	-154	9587	79
H(15)	6910	-182	8930	60
H(18)	8880	619	6259	43
H(21A)	9793	3081	5580	101
H(21B)	7770	3172	5599	101
H(21C)	8640	2522	5271	101
H(22A)	5915	1538	6576	77
H(22B)	6235	389	6569	77
H(22C)	5935	965	6174	77
H(17)	8367	1304	6984	42

Table A5. Hydrogen coordinates (x 10^{-4}) and isotropic displacement parameters ($Å^2$ x 10^3) for **4.11h**.

Bond	Angle (°)	Bond	Angle (°)
C(8)-C(1)-C(2)-C(3)	-0.9(3)	N(4)-C(5)-C(16)-O(16)	-177.8(2)
C(8)-C(1)-C(2)-C(9)	179.3(2)	C(6)-C(5)-C(16)-N(17)	-177.9(3)
C(1)-C(2)-C(3)-O(3)	-179.4(2)	N(4)-C(5)-C(16)-N(17)	2.7(3)
C(9)-C(2)-C(3)-O(3)	0.5(3)	N(17)-C(18)-C(19)-O(19)	-30.2(4)
C(1)-C(2)-C(3)-N(4)	0.9(3)	C(22)-C(18)-C(19)-O(19)	89.1(3)
C(9)-C(2)-C(3)-N(4)	-179.2(2)	N(17)-C(18)-C(19)-O(20)	153.9(2)
N(4)-C(5)-C(6)-C(7)	0.2(3)	C(22)-C(18)-C(19)-O(20)	-86.8(3)
C(16)-C(5)-C(6)-C(7)	-179.3(2)	C(7)-C(8)-N(4)-C(5)	0.5(3)
C(5)-C(6)-C(7)-C(8)	0.1(3)	C(1)-C(8)-N(4)-C(5)	-179.0(2)
C(6)-C(7)-C(8)-N(4)	-0.4(3)	C(7)-C(8)-N(4)-C(3)	179.5(2)
C(6)-C(7)-C(8)-C(1)	178.8(3)	C(1)-C(8)-N(4)-C(3)	0.0(2)
C(2)-C(1)-C(8)-C(7)	-178.6(4)	C(6)-C(5)-N(4)-C(8)	-0.4(2)
C(2)-C(1)-C(8)-N(4)	0.6(3)	C(16)-C(5)-N(4)-C(8)	179.0(2)
C(1)-C(2)-C(9)-C(10)	0.4(4)	C(6)-C(5)-N(4)-C(3)	-178.9(3)
C(3)-C(2)-C(9)-C(10)	-179.5(2)	C(16)-C(5)-N(4)-C(3)	0.6(4)
C(2)-C(9)-C(10)-C(15)	-110.2(3)	O(3)-C(3)-N(4)-C(8)	179.8(2)
C(2)-C(9)-C(10)-C(11)	70.1(3)	C(2)-C(3)-N(4)-C(8)	-0.5(2)
C(15)-C(10)-C(11)-C(12)	0.4(4)	O(3)-C(3)-N(4)-C(5)	-1.8(4)
C(9)-C(10)-C(11)-C(12)	-179.9(3)	C(2)-C(3)-N(4)-C(5)	177.9(3)
C(10)-C(11)-C(12)-C(13)	-0.7(5)	O(16)-C(16)-N(17)-C(18)	3.4(4)
C(11)-C(12)-C(13)-C(14)	0.7(5)	C(5)-C(16)-N(17)-C(18)	-177.14(18)
C(12)-C(13)-C(14)-C(15)	-0.4(5)	C(19)-C(18)-N(17)-C(16)	-72.5(3)
C(13)-C(14)-C(15)-C(10)	0.1(5)	C(22)-C(18)-N(17)-C(16)	168.0(2)
C(11)-C(10)-C(15)-C(14)	0.0(4)	O(19)-C(19)-O(20)-C(21)	-2.9(4)
C(9)-C(10)-C(15)-C(14)	-179.8(3)	C(18)-C(19)-O(20)-C(21)	173.1(2)
C(6)-C(5)-C(16)-O(16)	1.6(4)		

Table A6. Torsion angles (°) for **4.11h**.

REFERENCES FOR APPENDIX

1. Sheldrick, G. M.; University of Gottingen: 1997.

2. Sheldrick, G. M. *Acta Crystallogr., Sect. A* **1990**, *A46*, 467.